Calpain-1: Investigating Its Role in Murine Neutrophils.

by

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DECLARATION

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SUMMARY

Neutrophils are phagocytic white blood cells which act as the first line of defence against entry of foreign microorganisms. Neutrophils are recruited to their target site through the process of spreading, extravasation and phagocytosis involving complex signal transduction within the cells, which might include the activation of the cytosolic Ca^{2+} activated protease, calpain-1.

The work described here investigates the role of calpain-1 in regulating neutrophil functions such as spreading, trans-endothelial migration, chemotaxis, phagocytosis and Ca^{2+} signalling.

Through the work done at European Mutant Mouse Archive (EMMA), Oxford, and by using intracellular sperm injection (ICSI) of calpain-1 deleted gene from mice generated in the USA, and with a selective genotype breeding programme, a colony of homozygous calpain-1 KO mouse has been generated in Cardiff.

Homozygous calpain-1 KO neutrophils appeared to have a smaller surface spreading area and their recruitment into the peritoneal cavity of the mouse *in vivo* was disrupted. *In vitro* experiments showed significant defects in their ability to cross the ICAM-1 expressing endothelial cells in trans-endothelial migration assay. Disruption in this transmigration was only evident with ICAM-1 upregulated (TNF-treated) endothelial cells, suggesting a specific defect in the β2 integrin-ICAM-1 signalling process.

Calpain-1 absence did not affect signal transduction as neutrophils were able to signal cytosolic Ca^{2+} in response to β2 integrin engagement (C3bi-opsonised zymosan) and also to release intracellular Ca^{2+} store upon IP_3 uncaging. This showed that the IP_3 pathway in the cells was not affected by knocking-out calpain-1 and continued to be functional. The key signalling mechanisms from β2 integrin also remained intact and this is consistent with calpain-1 activation by Ca^{2+} being an important event in trans-endothelial migration.

In conclusion, calpain-1 absence has significantly affected the ability of neutrophils to undergo trans-endothelial migration and this effect is directed towards the event which happens downstream to the increase in cytosolic free Ca^{2+} concentration.

ABSTRACTS AND CONFERENCE PRESENTATIONS

Calpain-1: Their role in regulating neutrophils behaviour and characteristics

November 2010 **R Ishak,** S Dewitt and M B Hallett

Postgraduate Research Day, Cardiff University School of Medicine, Cardiff

The role of calpain-1 in regulating neutrophils behaviour and characteristics

September 2011 **R Ishak**

I3 IRG Annual Meeting, Thornhill Cardiff, Wales

Calpain-1: Investigating its role in murine neutrophils

April 2011 **R Ishak,** M B Hallett and S Dewitt

European Phagocyte Workshop, ESCI Conference, Crete, Greece

Calpain-1 is required for neutrophil trans-endothelial migration

November 2010 **R Ishak,** S Dewitt and M B Hallett

Postgraduate Research Day, Cardiff University School of Medicine, Cardiff

Investigating the role of calpain-1 in neutrophils

July 2010 **R Ishak**

I3 IRG Annual Meeting, Atlantic College, Wales

The therapeutic potential of novel calpain inhibitors for reducing inflammatory neutrophil trafficking

July 2010 M B Hallett, S Dewitt, **K J Lewis**, G Elumalai, R Ishak, I Laffafian, C Parr

I3 IRG Annual Meeting, Atlantic College, Wales

CONTENTS

ABBREVIATIONS LIST

7TM	7 Trans Membrane
AM	Acetotoxymethyl Ester
BSA	Bovine Serum Albumin
BSS	Balanced Salt Solution
C3	Complement Component 3
C3bi	Complement Component 3bi
C5a	Complement Component 5a
CAPN1	Calpain 1 (μ-calpain)
CAPN2	Calpain 2 (m-calpain)
CFU-G	Colony Forming Unit – Granulocyte
CFU-GEMM	Colony Forming Unit – Granulocyte, Erythroid, Monocyte, Megakaryocyte
CFU-GM	Colony Forming Unit – Granulocyte, Macrophage
CIF	Calcium Influx Factor
CLSM	Confocal Laser Scanning Microscope
CSF	Colony Stimulating Factor
CR3	Complement Receptor 3
CXCR1	CXC chemokine Receptor 1
CXCR2	CXC chemokine Receptor 2
DAG	Diacylglycerol
DMSO	Dimethyl Sulfoxide
EDTA	Ethylenediaminetetraacetic Acid
EGTA	Ethylene Glycol Tetraacetic Acid
ER	Endoplasmic Reticulum
ERM	Ezrin, Radixin, Moesin
FAK	Focal Adhesion Kinase
FCS	Foetal Calf Serum
FITC	Fluorescein isothiocyanate
fMLP	*N*-Formylmethionyl-leucyl-phenylalanine

G-CSF	Granulocyte–Colony Stimulating Factor
GFP	Green Fluorescent Protein
GM-CSF	Granulocyte Monocyte–Colony Stimulating factor
HBK	Hepes Buffered Kreb's Medium
HRP	Horseradish Peroxidase
ICAM-1	Inter-Cellular Adhesion Molecule 1
ICAM-2	Inter-Cellular Adhesion Molecule 2
IgG	Immunoglobulin G
IL-1	Interleukin 1
IL-3	Interleukin 3
IL-8	Interleukin 8
IP_3	Inositol 1,4,5-trisphosphate
kDa	kilodaltons
KO	Knock-out
LFA1	Lymphocyte function-associated antigen 1
MACS	Magnetic-activated cell sorting
NADPH	Nicotinamide adenine dinucleotide phosphate
NK	Natural Killer Cells
OPTI-MEM	OPTI-Modified Eagle's Medium
ORAI1	ORAI 1 Protein
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PIP2	Phosphatidylinositol 4,5-bisphosphate
PIP3	Phosphatidylinositol (3,4,5)-triphosphate
PKC	Protein Kinase C
PLCβ	Phospholipase C beta
PMN	Polymorphonuclear Leukocytes
RPM	Revolutions per minute
RPMI	Roswell Park Memorial Institute medium
SCF	Stem Cell Factor
SEM	Scanning Electron Microscope
SOCE	Store-Operated Calcium Entry

STIM1	Stromal interaction molecule 1
TBE	Tris/Borate/EDTA
TBS	Tris Buffered Saline
TNF-α	Tumour Necrosis Factor Alpha
TRIS	Tris(hydroxymethyl)aminomethane
UV	Ultra Violet
VCAM-1	Vascular-Cellular Adhesion Molecule 1
WT	Wild Type

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Chapter 1

Introduction

1.1. Introduction to Neutrophil

Neutrophils are phagocytic white blood cells (WBC) which act as the first line of defence in preventing the entry of any foreign microorganisms into the body. Neutrophils are grouped under the polymorphonuclear leukocytes (PMN) or also known as granulocytes, which defend the host against infections by means of a phagocytosis process that destroys invading microorganisms through its enzyme-rich lysosomes (Coico and Sunshine, 2009). Neutrophils are the major WBC populations which circulates the body in its spherical shape. It can be distinguished from the other WBC populations by its segmented nucleus of three to five lobules. Neutrophils are produced from the bone marrow and have a lifespan of up to 6 hours in the blood circulation. If the circulating blood neutrophils are not recruited they will undergo apoptosis in the liver or spleen, and when signals are generated the cells will perform their tasks before being removed from the body by other WBC.

In the event of inflammation, neutrophil will be the first group of cells arriving at the site of action to fight against microbial entry and to protect the host from infections. In this case, the chemoattractants released by the inflammatory reactions will prompt neutrophils to leave the blood vessels and direct the cells to the inflammation site. Neutrophils directed migration process towards this action site is known as chemotaxis. Recruitment of the cells is a multistep progression which begins with cell adhesion and spreading on the blood vessel endothelial cell lining before transmigrating by diapedesis to the inflammation site. Every step in this action is coordinated by inflammatory mediators such as formyl-Met-Leu-Phe (fMLP) and interleukin-8 (IL-8) (Lokuta et al., 2003).

1.2. Production of Neutrophils

1.2.1. Progenitor Cells

The production of neutrophils in the body involves several developmental stages and differentiation processes. The cells from the myeloid progenitor pools are generated from the stem cells which progressively develop into groups of committed cells that start with the colony forming unit-granulocyte, erythroid, monocyte, megakaryocyte (CFU-GEMM), then becomes the colony forming unit-granulocyte macrophages (CFU-GM) before subsequently developing into the CFU-G (colony forming unit-granulocyte). The term granulocyte is used generally to refer to neutrophils, but this would also include eosinophils and basophils. The hematopoietic stems cells that populated the bone marrow are pluripotent cells which are capable of replication as well as differentiation (Spangrude, 1994).

The CFU-GEMM is very similar in morphology to the stem cells but does not have the ability for self-replication. Their development continues with the formation of CFU-GM, and morphologically these cells are visibly different from the stem cells with larger size, more cytoplasm and noticeable nucleus (Metcalf, 1991). This CFU-GM is a mature progenitor myeloid cell that will then differentiate into monocytes or granulocytes before forming the CFU-G. The developmental process of both eosinophilic and basophilic granulocytes from progenitors is similar to neutrophils, although the commitment to differentiate either into neutrophils, eosinophils or basophils is probably established at the early progenitor stage (Morrison et al., 1995). The CFU-G will then progress to form the myeloblast which is the precursor cell for PMN.

1.2.2. Neutrophil Proliferation and Maturation

Neutrophil proliferation arises from a process called myelopoiesis which consists of three committed cell stages in the form of myeloblast, promyelocyte and myelocyte (Figure 1.2.2.1). The cells have the ability to divide and undergo several processes of division. Both myeloblasts and promyelocytes get through the division process once or twice, whereas the myelocytes undergo about three to four divisions throughout this whole process. The cell divisions continue with the formation of bands, and in the end differentiate into mature neutrophils (Bainton et al., 1971). Progressive maturation of granules in the cell is evident during the developmental stages. The azurophilic or primary granules can be distinguished during the promyelocytes stage which appears large in size. These granules contain among others, myeloperoxidase which is used to produce toxic bacteria-killing substance and lysozymes that helps to break down the bacterial cell wall (Weiss et al., 1978). Smaller granules also known as the specific or secondary granules can be visibly observed in the myelocytes and metamylocytes. The specific or secondary granules eventually develop into the neutrophilic bands before maturing into neutrophils. The mature neutrophils can be distinguished by their segmented nucleus, and at this stage only one-third of its granules are primary granules.

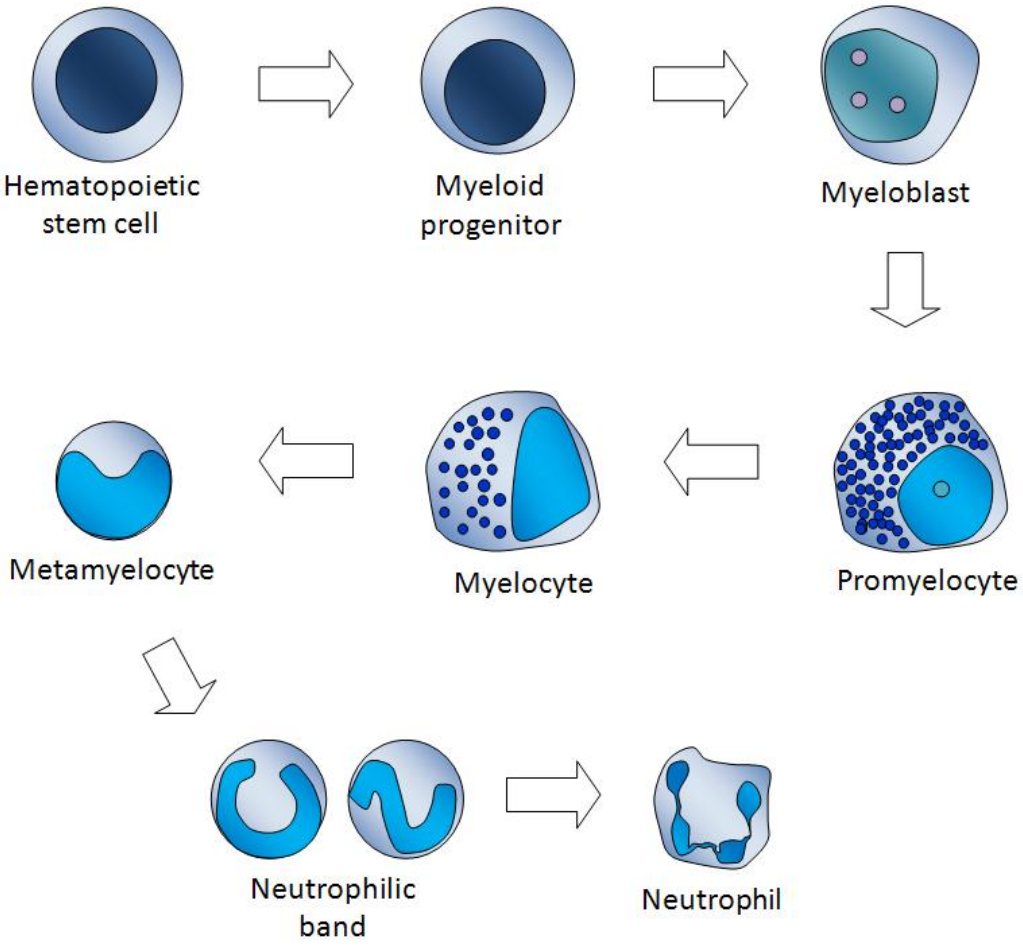


Figure 1.2.2.1: The developmental stages of neutrophils and their maturation process. This figure shows the proliferation and maturation stages of neutrophils from the hematopoietic stem cells.

1.3. Neutrophil Membrane Receptors

1.3.1. Complement Receptors

During the cellular division and maturation of the stem cells to become neutrophils, the membrane receptors were also being generated in the process. Complement receptors (CR) were occasionally detected in promyelocytes stage but more dominant at later stages. Receptors for the complement which consists of the CR_1 and CR_3 have been discovered on mature neutrophils (Glasser and Fiederlein, 1987). The CR_1 receptors have been extensively characterized in mature neutrophils but have low expression in the neutrophilic band stage. The CR_3 receptors were primarily seen on the promyelocyte cells and exist in over half of the metamyelocytes stage. The CR_3 receptors occupied almost all of the neutrophilic bands cells and in the mature neutrophils. In addition, the Fc receptor (FcR) for immunoglobulin G (IgG) was detected as early as the promyelocytes stage. Optimal FcR cell expression was detected during the neutrophilic bands and mature neutrophils stages.

During an inflammatory event, complement is activated and triggers a series of actions which then interacts with the complement receptors particularly CR_1 and CR_3. The activated complement intensifies the response and leads to the opsonisation and chemotaxis process. Once it is activated, the complement component C3, which is found in great quantity in the serum, is cleaved into C3a and C3b fragments. The C3b fragments were subsequently converted to C3bi. This C3bi fragment binds to the surfaces of the bacteria or fungi and enhance the phagocytosis process (Ross, 1989). The C3-binding protein has also been characterized on mouse neutrophils (Quigg et al., 1997).

1.3.1.1. Characteristics of the Complement Receptors

Over the years, several major complement receptors have been discovered namely CR_1, CR_2, CR_3 and CR_4/CR_5 (Becherer and Lambris, 1988; Ross, 1989). The CR_1 is found on the surfaces of many cells but most importantly is their presence on all of the phagocytes with high binding affinity towards C3b, which is the main opsonin fragment. In contrast, CR_2 is not found in any phagocytic cells although they are able to recognize C3bi fragments. CR_3 or also known by its CD (cluster of differentiation) designation as CD11b/CD18, which is found predominantly on all phagocytic cells, is another receptor that binds to C3bi fragments. This receptor is part of CD18 family with the same common β-subunit that is non-covalently connected to either one of the three α-subunit of CD11a, CD11b (CR_3) or CD11c (CR_4). The CR_4/CR_5 receptors (CD11c/CD18) which bind to the C3bi fragments are the third member of the CD18 family and could be found on the surfaces of some phagocytes and lymphocytes.

1.3.2. The Roles of C3bi and the Complement Receptors

The neutrophil phagocytosis process involves several steps starting with the contact and binding of target particles followed by its engulfment. After the first contact, receptors on the neutrophil interact with the opsonised particle through their cellular binding. These receptors send signals to the cells before forming its pseudopodia around the particles and completing phagocytosis process (Frank and Fries, 1991; Graham et al., 1989). Studies have proven that the opsonised particles binding and the signalling transmissions in the cells are a regulated function of phagocyte. PMN in a resting state have low binding capacity to C3bi-opsonised particles, but this is up-regulated in response to stimuli such as chemoattractants.

This effect happens when the intracellular pool of CR_1 and CR_3 is brought to the surface of the cells (O'Shea et al., 1985). However, this varies greatly depending on the activation state of the cells during purification as isolation methods could also enhance receptor expressions and binding affinity towards the opsonised particles. It is understood that C3bi-opsonised particles which binds to resting phagocytes could only be engulfed when cells are activated and at the same time having the phagocytic capability to do so. This signifies the importance of cell activation before performing their activity.

The C3bi fragment is reported to have a significant opsonin activity by binding to the CR_3 complement receptors on the neutrophil surfaces (Berger et al., 1984; Ross and Lambris, 1982; Shalit et al., 1987). This C3bi acts as a binding promoter for phagocytic cells such as neutrophils in particular, during which microorganisms or invading bacteria are coated with C3bi and allows activated neutrophils to recognize the foreign particle before prompting the phagocytosis reaction. The expression of CR_3 on neutrophils could be detected as early as the myelocyte stage, but fully expressed in the polymorphonuclear stages (Ross and Lambris 1982). PMN in whole blood samples have been found to have low numbers of CR_3 receptors together with low binding of C3bi on its surfaces. Activated neutrophils have demonstrated an increased CR_3 expression and proved to be an established cellular feature after being isolated. This phenomenon provides the opportunity to study the actions of isolated neutrophils and allows other experimental works to be done *in vitro*. Furthermore, the action and reaction of neutrophils beginning with opsonisation of foreign particles with C3bi, which is then presented to the activated neutrophils through CR_3 receptors binding, have greatly improved the efficiency of their phagocytosis process.

1.4. Neutrophil Chemoattractants

1.4.1. Major Chemotactic Stimulus for Neutrophils

In the event of pathogenic invasion or the removal of potentially harmful cell debris, neutrophils play an important role in the host defence mechanism. In normal physiological conditions, specific signalling molecules which are known as the chemoattractant will either be released by the foreign particles or generated by the host in order to direct the cells to the inflammation site (Zhelev and Alteraifi, 2002). Neutrophil progression from the blood circulation involves a defined interaction with membrane receptors on the cells. The cellular signal is generated and translated through a cascade of biochemical reaction within the cells which is referred to as signal transduction. It is well documented that neutrophils respond to several different chemoattractants which includes the complement-derived C5a molecule and the N-formylated peptides such as fMLP.

C5a is the cleaved product of the C5 complement component and acts as one of the major chemoattractants for neutrophils (Bohnsack et al., 1997; Huber-Lang et al., 2002). The production of C5a generates potent biological activities which subsequently cause the inflammatory reaction. Following the enzymatic reaction and the cascade of protein-protein interactions, C5a is produced through three complement activated pathways, the classical, lectin and alternative. One of the components that activate the classical pathway is the by-product from the inflammatory responses, whereas the lectin pathway is stimulated by the mannose production from the microbial surfaces. On the other hand, alternative pathway is either activated by the microorganisms as a whole or through direct contact with their by-products such as zymosan, lipopolysaccharide (LPS) or peptides. The cellular progression

from these three pathways continues by cleaving C5 into C5a which will then send signals from the target site. The production of C5a is not only restricted by these pathways as activated neutrophils contain neutral proteases that could cleave C5 into C5a. The C5a production enhanced the adhesion molecule expression and improved phagocytosis process (Guo and Ward, 2005). The C5a molecule acts as a targeting signal for neutrophil recruitment from blood circulation before reaching the inflammation sites. This initial signal stimulates neutrophil activities which will cause the cells to adhere and to migrate. The immediate arrival of neutrophils to their target location subsequently allows the cells to complete phagocytosis and limit harmful effect to the host. This reaction explains the significance of C5a production for neutrophils.

Another molecule which has shown to act as a chemotactic stimulus for neutrophil progression during the inflammatory response is interleukin-8 (IL-8). It was discovered that when properly stimulated, a lot of cells have the capability to produce IL-8 (Baggiolini and Clark-Lewis, 1992). This molecule is released by different tissues and cells upon exposure to the inflammatory stimuli and has also been recognized as a major chemoattractant for neutrophils. In a cellular study using a vessel wall model, the endothelial cell production of a specific 77-amino acid variant from IL-8 molecules were found to be a prerequisite for neutrophil transmigrations (Huber et al., 1991). Together with IL-8 antiserum, it has been shown that neutrophil transmigration ability was distinctly inhibited. It is believed that the secretion of IL-8 by the lipopolysaccharide-stimulated endothelial cells promotes the lectin adhesion molecule-1 (LECAM-1) shedding from neutrophils and at the same time up-regulating the β2-integrins molecules (Hammond et al., 1995). The IL-8 molecules also facilitate cellular adhesion and the transmigration progression. Cellular stimulation by using

IL-8 induced several main responses in neutrophils including their shape changes and directional movements (Baggiolini and Clark-Lewis, 1992). This response signified that these cells have a rapid reaction towards IL-8 and has comparable outcomes to the effects observed with C5a and fMLP. Therefore, the ability of IL-8 to recruit neutrophils during inflammatory reactions has proven it as a competent chemoattractant.

1.4.2. Receptors for Chemoattractants and G-protein Interactions

Previously, two different IL-8 receptors with seven transmembrane structures which function by means of G-proteins pathways have been identified through molecular cloning (Hammond et al., 1995; Stillie et al., 2009). Similarly, type 1 and type 2 IL-8 receptors have high binding affinities towards IL-8, except that the type 2 receptors bind to other related chemokines as well. This study showed that type 1 and type 2 IL-8 receptors are activated by IL-8 and directly involved with neutrophil chemotaxis. However, type 1 IL-8 receptors appeared to be the predominant mediator for chemotaxis function in neutrophils. The expression of the receptors for chemoattractants such as fMLP and C5a has also been successfully characterized. The structures of both human C5a and N-formyl peptide receptor have been identified and share a rather striking resemblance (Boulay et al., 1991). These receptors have been identified as members of the seven transmembrane domain of GTPase-coupled receptor superfamily (Guo and Ward, 2005). The third intracellular loop of these seven transmembrane receptors has been proposed to be a G-protein binding domain for N-formyl peptide and C5a. This G-protein domain transmits signals to the cells interior which acts as intermediary between receptors on the surface of the cells and the effector enzymes that are responsible for generating the second messengers.

Generation of the second messengers modulate neutrophil function by dramatically increasing their level as a response to the chemoattractant and receptors binding. The generation of the second messengers includes Ca^{2+} and inositol triphosphate (IP_3) (Davies-Cox et al., 2001; Ferris and Snyder, 1992; Omann et al., 1987). Increases in concentration of free intracellular Ca^{2+} begins rapidly after being induced by chemoattractants. This process involves at least two mechanisms of action with intracellular Ca^{2+} storage released which is mediated by the IP_3 and an influx of extracellular Ca^{2+}. The increase in intracellular Ca^{2+} level induced by the chemoattractant slowly returns to baseline level in between 5 to 15 minutes. This response involves the protein kinase C (PKC) and/or cyclic adenosine monophosphate-dependent protein kinase A (cAMP-PKA). PKC triggers the activation of Ca^{2+}-ATPase on the plasma membrane and then pumps Ca^{2+} out of the cell. Furthermore, both PKC and PKA could also inhibit the production of IP_3 and subsequently impede further release of intracellular Ca^{2+} storage.

1.4.3. The Roles of Chemoattractants in Neutrophil Chemotaxis

The cellular signalling during neutrophil chemotaxis involves interactions between the chemoattractants and plasma membrane receptors, which is followed by cell adhesion and reversible construction of the cytoskeleton element. The transmitted signal will prompt the cells to undergo rapid morphological changes and allows it to transform from spherical shape to flattened (Dewitt and Hallett, 2007) (Figure 1.4.3.1). Once stimulated by the chemoattractants, neutrophils adhered onto the endothelial cells of the blood vessels form pseudopodia which are then extended towards the chemoattractant gradients (Cassimeris et al., 1990). Neutrophil migration involves complex cyclical events of extending and

retracting their lamellipodia and uropodia respectively, towards the chemoattractant gradients. Neutrophils adhere reversibly to the substratum allowing the cells to travel along the surface of the endothelial cells (Pierini et al., 2003).

Neutrophil movement involves globular proteins of the microfilament cytoskeleton called actin. After neutrophils are stimulated by the chemoattractant, monomers of globular actin (G-actin) are polymerized into actin filaments (F-actin) which is associated with shape changes and migration rates. This reversible actin assembly and networking is regulated by an array of actin-binding proteins (Omann et al. 1987). Neutrophil progression could either be in chemotactic, chemokinetic or random migration behaviour. The non-directional motile reactions during random migration could happen without the presence of chemoattractant or even during chemokinetic migration. In contrast, the chemotactic response moves in a directional manner towards the chemoattractant gradients. It is likely that these three categories of cellular motility are intermittently involved in neutrophil mobilization to their target sites.

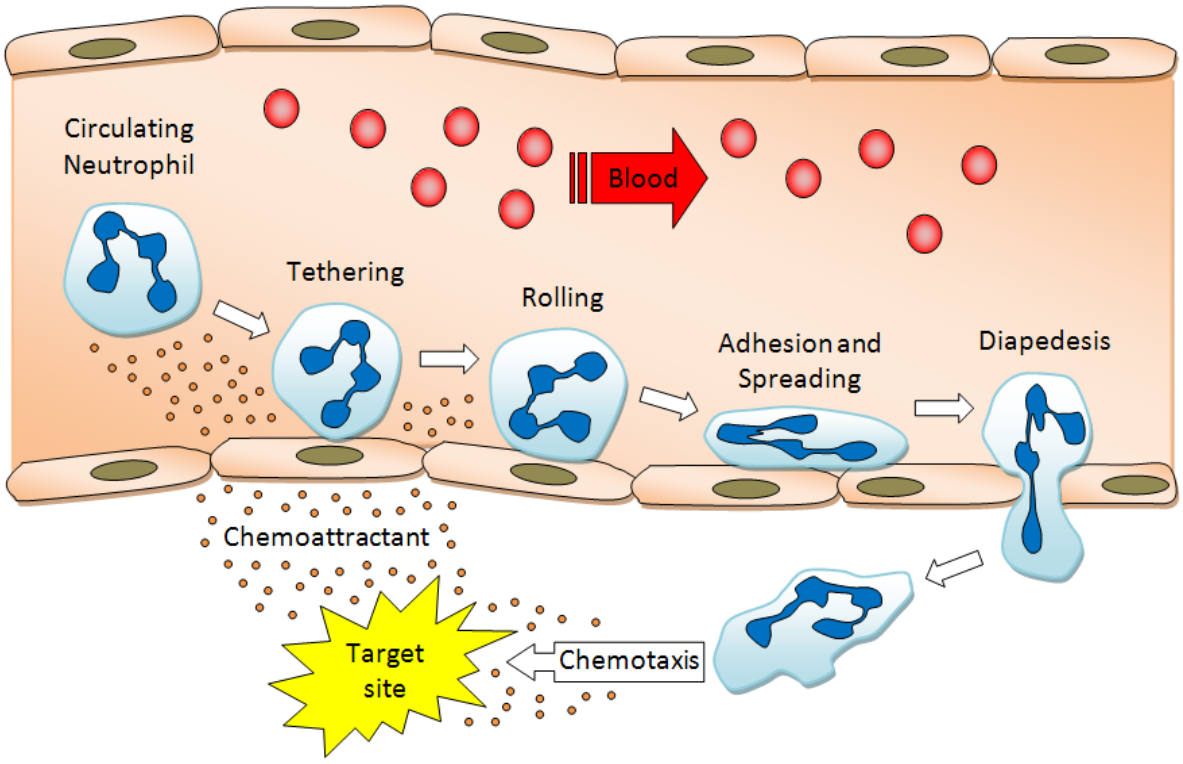


Figure 1.4.3.1: The physiological behaviour of neutrophils in response to chemoattractant signals. Neutrophil tethering, rolling, adhesion, spreading, diapedesis (transmigration) and chemotaxis.

1.4.4. Chemoattractant Activation and Cytoskeletal Changes

The reversible assembly of G-actin structure into F-actin allows neutrophils to explore their extracellular surroundings. The majority of the total actin in unstimulated neutrophils exists as G-actin and this proportional number rapidly increases to the filamentous F-actin form to about two-fold after the chemoattractant stimulations (Muller, 2011; Southwick et al., 1989). This exposure to chemoattractant causes sudden fluctuations in the actin polymerization and depolymerization that happen in just a few seconds. The link between chemoattractant the receptor-ligand complex and the neutrophil cytoskeleton are immediately noticeable through the reactions which involve the F-actin.

Migrating neutrophils in response to the presence of chemoattractant travel while being adhered to the extracellular matrices. This reaction sends multiple signals which will subsequently cause polymerization of the actin. This actin polymerization can be detected during neutrophil adherence to their exterior surface. Adhered neutrophils are shown to have different F-actin responses in comparison to those elicited by the chemoattractant (Cassimeris et al. 1990). This assembly of F-actin in chemoattractant-stimulated neutrophils appeared to be more rapid as compared to the adherence-induced actin polymerization. The actin assembly is essential for the pseudopodia extension and this could be observed by adding chemoattractant to the cells which would double the level of F-actin within the newly extended pseudopodia (Pierini et al. 2003). The pseudopodia extension, as well as the actin polymerization, could only be achieved and maintained through the continuous presence of chemoattractant. Removal of chemoattractant stimulus eventually caused the pseudopodia to withdraw and eventually diminished the F-actin polymerization.

1.5. Neutrophil Phagocytic Process

1.5.1. Phagosome and Lysosome Functions

In any incident of microbial invasion, cellular signals in the form of chemoattractants are transmitted and begin to recruit circulating neutrophils from the blood. The activated neutrophils adhere to the endothelial cells lining the blood vessels and leave the blood circulation by transmigrating through the cell junctions towards the signal source. As neutrophils migrate, the surface of the microbe will be opsonised with C3bi and antibody for recognition as well as for the eventual phagocytosis process. By having receptors that are distinctly intended for binding with C3bi and antibody, the opsonisation process will help the cells to recognize the microbe through their receptor-mediated interaction. As a result, the microbe is rapidly engulfed and digested by the neutrophil.

Phagocytosis is the process that takes up opsonised particles into phagocytic vesicles called phagosome and leads to ingestion of the particles. This event involves attachment of particles to the receptors on the cells, resulting in engulfment. Phagocytosis begins with binding of the receptor-ligand between neutrophils and the particles which will activate the ingestion phase involving the actin-binding proteins (Lee et al., 2003; Segal, 2005). The actin microfilament which lies beneath the cytoplasm sections at the point of contact with the particles will then polymerize. As a result of this microfilament attachment, polymerization eventually causes the plasma membrane of the cells to bundle at the site of contact. As a result, the particle is enclosed by membranes to create a new particle-membrane contact site and followed with pseudopodia formation before eventually forming the phagosome. The cells contain lysosomes or secretory granules in the cytoplasm which contain what is

known as ‘digestive’ substances to help internalize the particles (Hallett and Dewitt, 2007). These lysosomes will fuse to the phagosomal membrane to form the phagolysosome which also provides additional membrane locally. This may be a part of the mechanism that provides extra membranes to engulf particles during phagocytosis.

1.5.2. Vesicle Fusion and Killing Process

In the phagocytic process, all events with regard to digesting the particles happen within the phagolysosomes. Without lysosomal fusion, phagocytosed particles, particularly the larger ones are incompletely internalized. However, before the particles are enclosed by the phagosome, the granule contents may perhaps accumulate at the contact sites or seep into the extracellular spaces (Haas, 2007; MacRae et al., 1980). The leakage of the potent hydrolases outside of the sealed phagosome may underlie the damaging effects due to the inflammatory reactions. Small amounts of neutrophilic granule derived myeloperoxidase are also released during phagocytosis and appear in abundance after the closure of the individually formed phagosomes. It is believed that influx of this granule contents in the phagosomes is completed through a continuous channel formed by the lysosomes fusion with the phagosomal membrane before the digestive substance is added to the phagosomes accordingly. Therefore, removal of the engulfed particles could only happen after the lysosomal granules have fused together with the phagosome.

1.6. The Roles of Ca^{2+} Signalling in Neutrophils

1.6.1. Ca^{2+} Storage and Release

The importance of cytosolic-free Ca^{2+} and its effects on neutrophils has been well documented through the years. Intracellular Ca^{2+} is important for neutrophil to function through its participation in a number of roles that include cell adhesion and shape change. Stimuli which either engage seven transmembrane receptors or act through cross-linking receptors, release stored intracellular Ca^{2+} (Pettit and Hallett, 1998). Two distinctive but separate locations for Ca^{2+} storage and release have been identified within the neutrophil. One of the sites was found in the cells periphery and lies under the plasma membrane. Ca^{2+} from this location is released locally upon adherence and cross-linking stimulus through integrin engagement. During chemotaxis, these Ca^{2+} stores move to the leading edge which demonstrates their mobility and role in the cell's movement. The other site for Ca^{2+} storage was in the juxtanuclear space which is located in the center of the cell, normally close to and between the nuclear lobes. Ca^{2+} is released from this site by fMLP stimulation. This finding suggests that the presence of these two distinctive Ca^{2+} storage and release sites requires the generation of separate intracellular messengers.

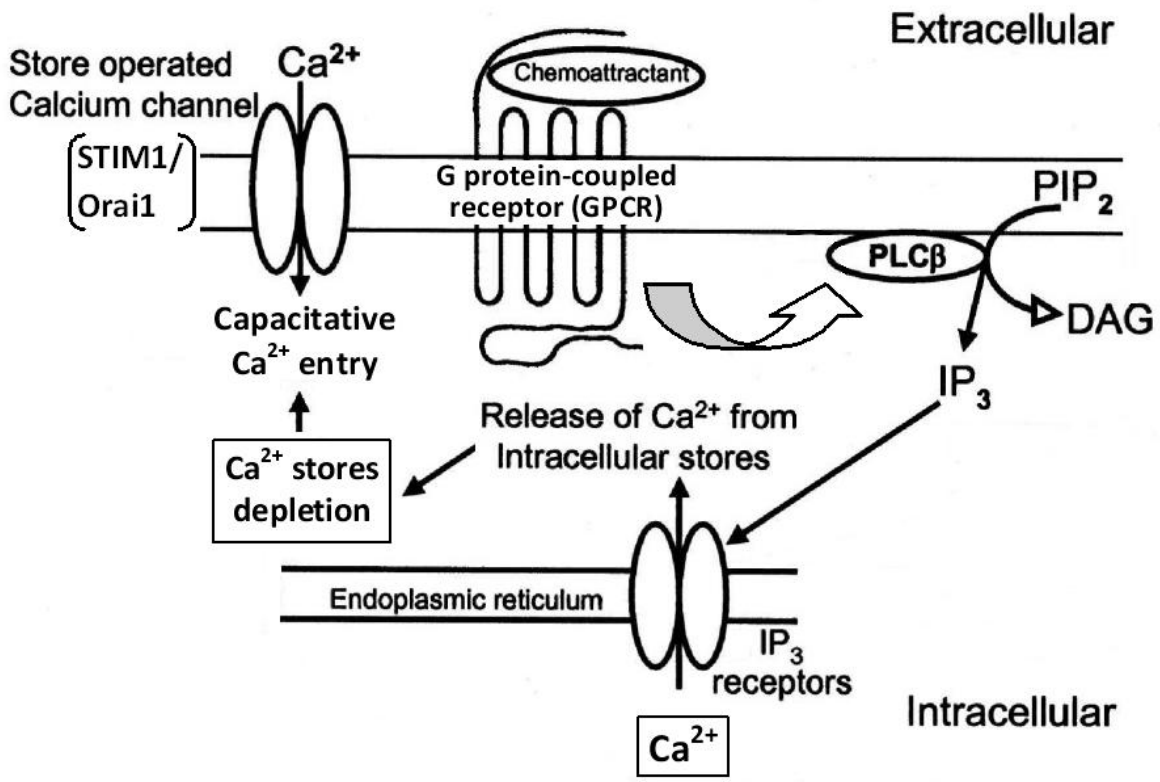
The release and rise of Ca^{2+} levels in neutrophils occurs through different pathways. The cell activation that occurs through chemotactic stimuli leads to the release of Ca^{2+} from intracellular storage together with an influx of extracellular Ca^{2+} in an almost simultaneous manner (Davies and Hallett, 1995). Immune complexes signalling via Fc receptor binding and cross-linkage on neutrophils also increase the concentration of cytosolic-free Ca^{2+}, and this is also a two-phase increase in the cytosolic-free Ca^{2+} level but both of these phases show

different responses to fMLP stimulation. It was noted that the first Ca^{2+} rise is a result of Ca^{2+} release from intracellular storage, but from an apparently different location to that released by chemoattractants (Jaconi et al., 1991). Furthermore, the second phase increase, which results from transmembrane Ca^{2+} influx, is delayed compared to the fMLP route. The immune complex pathways have different mechanisms for Ca^{2+} channel opening to the one used by fMLP. In contrast to the fMLP stimulus route, transmembrane Ca^{2+} influx through immune complex pathways were not driven by the emptying process of intracellular Ca^{2+} storage. It has been proposed that Ca^{2+} channel opening via fMLP is controlled by what is referred to as "Ca^{2+} influx factor" that is freed during intracellular Ca^{2+} release. In contrast, Fc portion binding to its receptors could possibly open up Ca^{2+} channels via a direct route and the intracellular Ca^{2+} release through this pathway do not have the Ca^{2+} influx factor.

Another common pathway that increases the Ca^{2+} concentration is generated by the phospholipase C (PLC) enzyme. The activation of PLC is regulated through G protein-coupled receptors (Omann et al., 1987). PLC hydrolyses a component on the cell membrane called PIP_2 (phosphatidylinositol 4,5-bisphosphate) to form IP_3 (inositol 1,4,5-trisphosphate) and DAG (diacylglycerol), which are the intracellular second messengers (Hillson et al., 2006). IP_3 has been implicated with the release of intracellular Ca^{2+} stores release by diffusing through the cytoplasm to the endoplasmic reticulum (ER). IP_3 binds to the IP_3 receptors on the surface of ER which causes the opening of Ca^{2+} channel and releasing the Ca^{2+} stores (Berridge et al., 2000; Bootman et al., 2002; Smith et al., 1990). This is then followed by the influx of Ca^{2+} across the plasma membrane which is mediated by the depletion of intracellular Ca^{2+} storage through a mechanism known as capacitative Ca^{2+} entry (Berridge, 1995; Demaurex et al., 1994; Kiselyov et al., 1998) (Figure 1.6.1.1).

Human neutrophils have been shown to have both "conventional" stationary Ca^{2+} "puffs" and waves of global Ca^{2+} signals spreading across the cells upon stimulation with formylated peptide or with cytosolic IP_3 uncaging stimulation (Hillson and Hallett, 2007). Detection of the Ca^{2+} puffs suggested that neutrophils have only one Ca^{2+} release site in each cell. The existence of a single organelle may be characterized by the discovery of the vestigial Golgi or a nuclear envelope projection that is located next to the nucleus. Neutrophils have very low numbers of IP_3 sensitive receptors for Ca^{2+} release and Ca^{2+} puffs were probably generated through this particular mechanism. In addition, the elevation of cytosolic free Ca^{2+} has been identified as a key signalling component in neutrophils, particularly in their shape changing phenomenon from spherical to the flattened form. It is also believed that this Ca^{2+} signal is responsible for many other responses within the cells.

ORAI calcium release-activated calcium modulator 1 (Orai1) and stromal interaction molecule 1 (STIM1) were reported to function as a Ca^{2+} channel subunit which facilitates the store-operated Ca^{2+} entry (SOCE). STIM1 has been implicated as the sensor for Ca^{2+}-release activated Ca^{2+} (CRAC) channel opening upon Ca^{2+} storage depletion, which is followed by the influx of Ca^{2+} (Zhang et al., 2005). It has been suggested that SOCE might regulate neutrophil activation during their recruitment to the inflammation sites via Orai1 (Schaff et al., 2009). This finding also implies that Ca^{2+} influx via Orai1 is the major SOCE which works together with intracellular Ca^{2+} store release in neutrophil arrest and migration. During phagocytosis, it has been demonstrated that Ca^{2+} influx is facilitated by Orai1 and STIM1 (Steinckwich et al., 2011). It was proposed that the phagocytosis process requires extracellular Ca^{2+} entry and intracellular Ca^{2+} store depletion for the internalization phase. Thus, Orai1 and STIM1 have been associated with the elusive capacitative Ca^{2+} influx mechanism (Figure 1.6.1.1).



(Berridge, 1995; Clapham, 2007; Cope, 2002; Ley et al., 2007)

Figure 1.6.1.1: The present understanding of Ca^{2+} mediated signaling. This figure illustrates the present understanding of Ca^{2+}-mediated signalling, with emerging themes related to the Ca^{2+} binding proteins and Ca^{2+} entry across the plasma membrane.

1.6.2. Ca^{2+} Signals in Adhesion and Spreading

Neutrophil adhesion and spreading are the early steps in the immune response that precede the extravasation process. Repeated increase of cytosolic free Ca^{2+} has been documented in neutrophils undergoing the adhesion process through CD11b/CD18 integrin engagement (Pettit and Hallett, 1997; Schaff et al., 2008). The increasing level of Ca^{2+} via this pathway has a time-dependent effect, with full concentration achieved after about 100 seconds. The next pulse of Ca^{2+} influx could only have a level that is greater or equal to the previous reading if sufficient time elapsed before the next signal. This effect is as opposed to the level of Ca^{2+} that peaks before 100 seconds which could not reach the same height. The integrin engagement may produce signals for Ca^{2+} release through receptors on its storage site and consequently cause the influx of Ca^{2+} in the cells. The signals for Ca^{2+} influx and initiation of the next Ca^{2+} influx pulse occurs with continuous integrin molecule engagement on the cell surface. Respectively, these responses suggested that integrin engagement needs to reach a certain level to signal Ca^{2+} influx and sufficient integrin molecules need to be available to generate the next Ca^{2+} influx pulse. These attributes point to cytoskeleton connection during Ca^{2+} storage release at the integrin engagement sites and the signals for Ca^{2+} influx.

It has been reported that a brief increase in the cytosolic free Ca^{2+} concentration has been detected when neutrophils spreads (Brasen et al., 2010; Kruskal et al., 1986). The cell's ability to spread depends on two factors, the rise in cytosolic free Ca^{2+} and the nature of the surface for spreading. Photolytic uncaging of cytosolic caged Ca^{2+} in neutrophils gives rise to elevated Ca^{2+} levels but has different spreading effects in the non-adherent and in integrin-

engaging adherent cells. The rapid morphological change that is identified as spreading was observed with cells engaged to a CD11b/CD18 integrin but not in the non-adherent cells on plain glass (Pettit and Hallett 1998). It is believed the integrin engagement acts as the second signal and that cytosolic free Ca^{2+} rise is not the only signal for neutrophils to change their shapes. After integrin engagement, changes in global Ca^{2+} are the trigger for rapid cell spreading. This suggests that the rise in cytosolic free Ca^{2+} is a requisite but not the solitary signal for cell spreading. This mechanism is an essential aspect in neutrophil extravasation and it has been shown that Ca^{2+} exhaustions or buffering inhibits their ability to migrate on fibronectin by preventing the release of adhesion at the cells rear of the cells (Cox and Huttenlocher, 1998).

The need for neutrophils to spread and establish firm adhesion on endothelial cells for transmigration is very prominent. By looking at flattened or spread neutrophils, there is in fact more than a 100% surface area increased when compared to the wrinkled spherical shaped cells. The presence of a membrane reservoir is believed to allow neutrophils to expand (Kruskal et al., 1986). Cell spreading is accompanied by unwrinkling the non-elastic plasma membrane, creating a “stretching” effect that allows the cells to flatten before firmly adhering to the endothelial cells. It has also been shown that the cells flattening rate could be influenced by controlling their cytosolic Ca^{2+} levels through IP_3 uncaging (Dewitt and Hallett 2007). Thus, it is believed that this effect is associated with the activity of Ca^{2+}-activated protease enzyme called calpain. Calpain activation by means of Ca^{2+} rise may cleave some cytoskeletal protein substrates within the cells before unbinding the cytoskeleton anchors to plasma membrane which then provides additional membrane for cell expansion and spreading.

1.6.3. Ca^{2+} Requirement in Chemotaxis and Phagocytosis

In neutrophils, chemotaxis and phagocytosis are the other major functions in their host defence task. These functions are primarily instigated by the chemoattractant together with the presence of microorganisms during cellular invasion. It has been shown that the co-existence of two phagocytic mechanisms is correlated with the elevation of intracellular Ca^{2+} concentration in the cells (Lew et al., 1985). In the phagocytosis process which is mediated through C3b/bi contact, the involvement of intracellular Ca^{2+} is considered to be secondary and not necessarily related to the entire process. However, it is thought that independent machinery which facilitates the restructuring of the contractile proteins in order to engulf the particle does exist. When the phagocytosis process is initiated by IgG molecules through Fc receptor activation, neutrophils have an intracellular Ca^{2+} concentration dependency which needs to be at a physiological level. This mechanism may perhaps require localized changes in the intracellular Ca^{2+} concentration near to the plasma membrane which could also be the switch for the phagocytic engulfment process.

Cytosolic free Ca^{2+} transients have also been measured in human neutrophils going through rapid directed movement following a chemoattractant gradient. By using the sheep erythrocytes opsonised with either IgG and/or IgM to generate the C5a chemoattractant, recurring episodes of transient elevations in the cytosolic free Ca^{2+} concentration during chemotaxis and phagocytosis could be detected in human neutrophils loaded with the Ca^{2+} sensitive Fura-2 indicator (Marks and Maxfield, 1990). Rises in localized cytosolic free Ca^{2+} transients were observed in the periphagosomal areas of the cells during phagocytosis and the rise in Ca^{2+} level is dependent on the nature of the opsonin used. However, the localized

increased in cytosolic free Ca^{2+} concentration was not present in any parts of the cells undergoing chemotaxis. In general, changes in cytosolic free Ca^{2+} and its intracellular distribution occur rapidly upon stimulation.

It has been described that Ca^{2+} signals in neutrophils could be distinguished by two chronologically separated mechanisms. The first Ca^{2+} signal can be detected at the moment of integrin engagement and the second signifies the global Ca^{2+} signal which is driven by Ca^{2+} influx observed at the time of phagosome closure. Neutrophils presented with C3bi-opsonised zymosan particles using a micropipette have shown the sequential Ca^{2+} signals which accompany phagocytosis with the first one recorded at the time of particle contact and the second one measured as the cells completed the engulfment (Dewitt et al., 2003). However, only the second Ca^{2+} signal is linked with the oxidative action in the phagosome and this signal is not confined just to the phagosome region. Therefore, increase of the first cytosolic free Ca^{2+} signal is deemed to be inadequate for the oxidase activation and other effects, but it is thought that a slower action is also involved during the process. It is possible that this slower action is involved in the mechanisms for oxidative component formation in the phagosomal membrane which is completed by the time of the second Ca^{2+} signal.

In a proposed model, the initial contact made by human neutrophils undergoing phagocytosis of C3bi-opsonised particles results in the cross-linking of specific molecules on the cell surface called β2 integrins, which cause localized Ca^{2+} release from its storage site (Dewitt and Hallett, 2002). This reaction is then followed with the formation of a phagocytic "cup" at the contact point with the particles. However, engulfment of particles is limited by the number of β2 integrin molecules tethered to the cytoskeleton. Depletion of localized

stored Ca^{2+} generates a diffusible signal to the plasma membrane and eventually opens up the Ca^{2+} channels. As a result, the rise of Ca^{2+} level can again be seen in the whole cell and this influx of Ca^{2+} subsequently causes activation of an enzyme called calpain. Activated calpain will eventually release the outlying β2 integrin molecules from their tethers before allowing the cells to move towards the bound opsonised particle and complete the whole phagocytosis process.

1.6.4. Potential Regulatory Enzyme for Neutrophils

The role of Ca^{2+} signalling in neutrophils as reported in many of the previous studies highlights the importance of this function. One of the notable events is the possible role played by the enzyme calpain which upon activation by means of increased Ca^{2+} level allows the cells to complete their tasks. It is believed that calpain is one of the main components in neutrophils and its interaction with other molecules facilitates the cell's capacity to function at an optimum level. It has been reported that calpain is engaged during the migration process in most cell types including neutrophils (Saez et al., 2006). There is much evidence, data and proposed ideas as to how calpain might regulate the behaviour of neutrophils. Calpain may have roles in the adhesion and morphological changes of neutrophils from the resting spherical shape to their spreading form. Similarly, calpain is also thought to be involved in neutrophil detachment from the extracellular matrix by acting on the adhesion complex which would liberate the cells and allow them to move. Although calpain is believed to have a major role in neutrophils, there have been several conflicting reports on the role of this enzyme. Thus, the need to have a better understanding of how calpain regulates neutrophil functions is important if it is to be considered as a therapeutic target.

1.7. Calpain Properties and Systems

1.7.1. General Properties of the Key Calpains and Their Endogenous Inhibitor

Calpains are Ca^{2+}-dependent cysteine proteases that function as regulatory enzymes to many cells. Two of the key calpains that have been identified are μ-calpain, also known as calpain-1, and m-calpain or calpain-2. The name μ-calpain and m-calpain referred to the Ca^{2+} concentration needed to activate these enzymes, with μ-calpain requires Ca^{2+} in micromolar whereas m-calpain needs Ca^{2+} in the millimolar range (Goll et al., 2003). This enzyme exists as heterodimers that consist of two subunits, the large catalytic subunits (of either calpain-1 or calpain-2) and a common smaller regulatory subunit (also referred to as calpain-4) (Figure 1.7.1.1). The molecular mass of the large subunit for both calpain-1 and calpain-2 is about 80kDa with calpain-1 being slightly larger than calpain-2. The smaller regulatory subunit is identical in both calpain-1 and calpain-2 and has a molecular mass of approximately 28kDa. Together, the large and small subunits of the calpain structure contain six domains that have different functions (Franco and Huttenlocher, 2005). Based on the amino acid sequence, the large 80kDa subunit is divided into four different domains namely domain I, II, III and IV (Figure 1.7.1.1). Domain I undergoes autolysis upon interactions with the smaller 28kDa subunit and is important for the enzyme stability. Domain II is divided into sub-domain IIa and IIb, and is the protease activity sites, whereas domain III is the C2-like domain where the phosphorylation and phospholipid binding takes place. As for domain IV, it has five EF-hand motifs for Ca^{2+} binding as well as dimerization with the smaller regulatory subunit. In addition, the smaller 28kDa subunit has domain V which is rich with glycine and highly flexible, together with domain VI that has five EF-hand motifs with the last motif interacting with the large 80kDa subunit.

Presently, calpastatin is the only known endogenous inhibitor for calpain and can be found in various tissues (Saez et al. 2006). It has domain structures that bind specifically to calpain subsequently inactivating the enzyme. Previous studies have demonstrated that Ca^{2+} is required by calpastatin to either bind or inhibit calpain and that calpastatin inhibitions are a reversible process. The calpastatin inhibits calpain only in the presence of physiological Ca^{2+} concentration, and overexpression of calpastatin in the cells showed decrease calpain activity but this condition alone is not enough to inactivate calpain (Goll et al. 2003). It has been shown that Ca^{2+} requirement for calpastatin to bind to calpains depends on the calpain molecules itself.

Data from previous biochemical studies implied that calpastatin may perhaps have inclination to bind to Ca^{2+}-activated calpain and suggested the existence of an attenuation mechanism which decreases calpain activity. Furthermore, it appears that calpain required a significantly higher Ca^{2+} concentration to activate its proteolytic activity as compared to the level needed for the enzyme to bind to calpastatin (Friedrich, 2004). Requirement for this higher Ca^{2+} level for calpain proteolytic activity also acts as safety system which is dedicated to preventing any possible destructive hyperactivity of the enzymes. The increasing concentration of Ca^{2+} in cells initially causes the binding of calpastatin to calpain at the same localization where it induces calpain proteolytic activities. This elevated Ca^{2+} concentration in the cells will initiate the autolysis process of calpain before eventually exposing its active site at the centre of the molecule before immediately becoming inactivated by the bound calpastatin through a competitive mechanism of action.

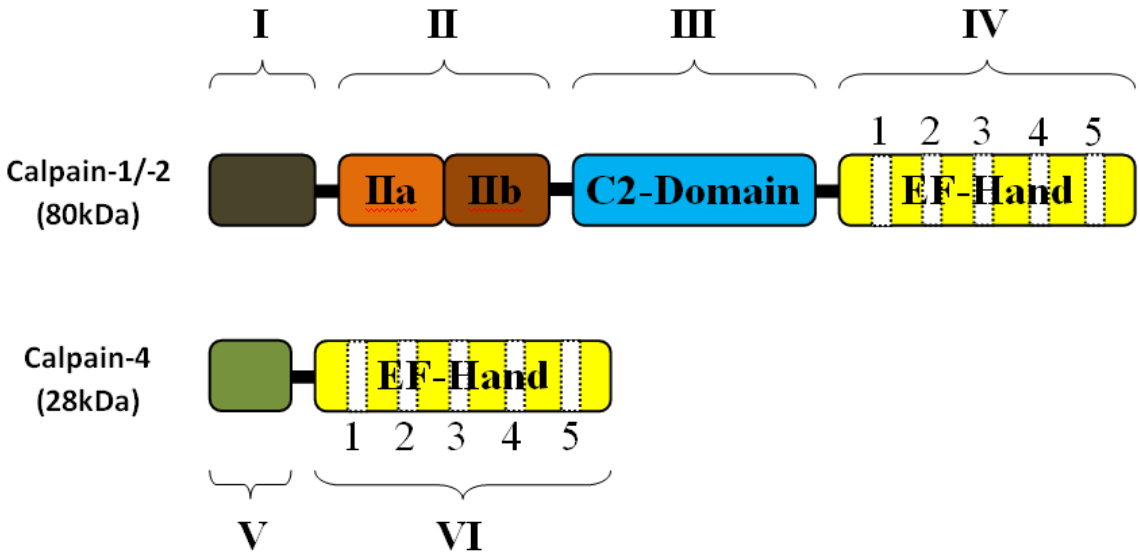


Figure 1.7.1.1: Calpain structure and its domain.

1.7.2. Localization of Calpain and Calpastatin in the Tissues

Earlier studies have shown that both calpain-1 and calpain-2 are exclusively localized in the cytoplasm but the distribution depends on the type of cells. Calpains are localized at the cytosol and may relocate to the peripheral sites from scattered distribution in response to cellular signals that could probably be the influx of Ca^{2+} (Goll et al. 2003). In the unstimulated cells, both of the calpain 28kDa and 80kDa subunits are distributed in the cytoplasm and show slightly higher fractions around the nucleus region (Gil-Parrado et al., 2003). Calpain subunits are redistributed to the plasma membranes after stimulation with the Ca^{2+} ionophore. Both calpain and calpastatin appeared to be co-localized at the same site within the cells. Like calpain, calpastatin is also distributed in the cytoplasm where it is located close to the nucleus of unstimulated cells, but upon stimulation still remains at the same location. It has been suggested that significant percentages of calpain are associated with the subcellular structure such as actin filaments of the cells cytoskeleton. The calpain-1 activity is primarily confined in the cytosol of the cells, whereas most of calpain-2 activity is associated with the membrane. However, *in vitro* studies in rat myoblasts showed that the presence of calpain-2 outside the cells and suggest its extracellular role in regulating cell to cell interactions (Sazontova et al., 1999).

1.7.3. Potential Substrates for Calpain

A large number of proteins have been recognized as potential calpain-1 substrates. Some of these substrates could become completely degraded by calpain or partially cleaved, and result in the formation of stable protein fragments with different functions to the

original form. Partial proteolysis of some of the substrates might be related to a very specific molecular recognition. Generally, most of the substrate is categorized into four different groups namely the cytoskeletal proteins, the membrane associated proteins, transcription factors and kinases and phosphatases (Goll et al. 2003). At present, there is no consensus on the calpain proteolysis sequence or even the exact cleaving location for these substrates. However, it appears that most of the cytoskeletal proteins such as talin, vinculin, spectrin, filamin and ezrin which act as the anchor between cell cytoskeleton and plasma membranes are rapidly cleaved by calpain and consequently breaks the binding that holds the cell shape together.

It has been suggested that the ability of calpain to cleave protein kinase C (PKC) can be detected in the signal transduction pathways (Ng-Sikorski et al., 1991). However, *in vivo* studies have failed to recognize the specific calpain substrate which is related to this signalling event. The role of calpain in signalling transduction is mainly associated with its function during the interaction between the plasma membrane and cytoskeleton (Figure 1.7.3.1). It has been demonstrated that calpain is involved with the integrin mediated signals through its specific cleavage action of the β-integrin family which severs any interactions before disassembling the cells focal adhesion molecule (Pfaff et al., 1999). However, calpain proteolysis actions and its specificity towards focal adhesion components vary between different cell types.

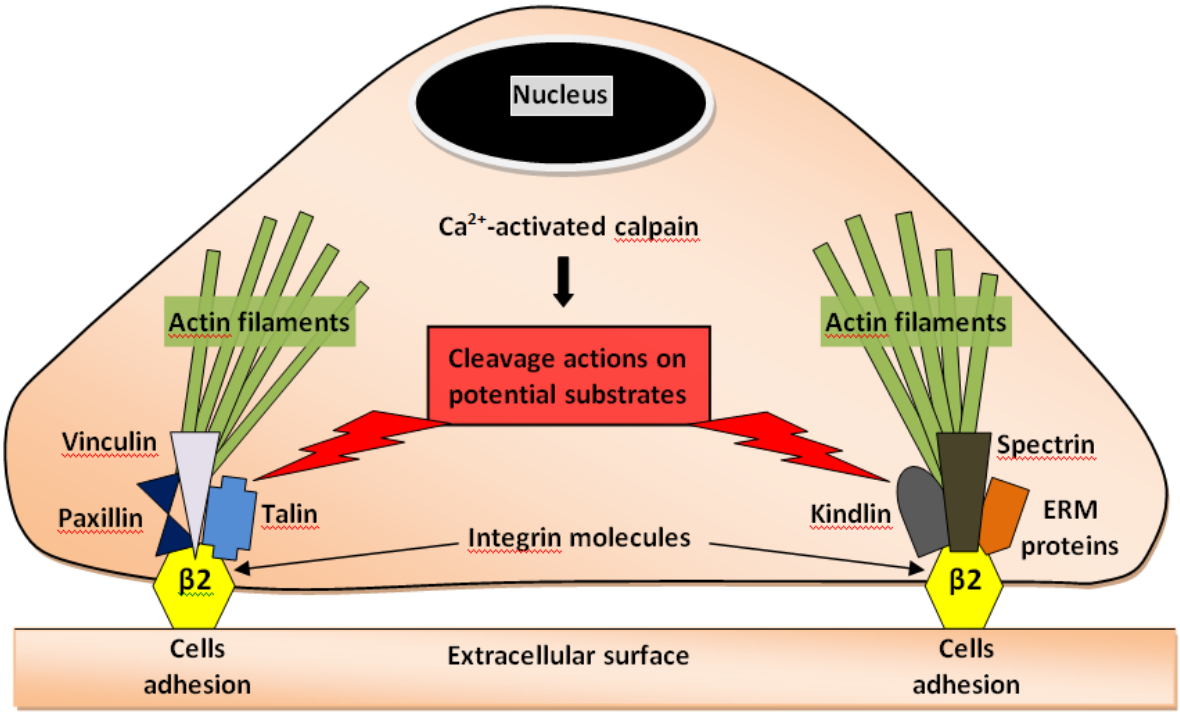


Figure 1.7.3.1: Calpain cleavage actions on the potential substrates. This figure shows the potential substrates for calpain-1 that link neutrophil β2-integrin molecules with the actin filaments bundles.

1.7.4. Calpain Major Functions

Calpain has been implicated in several major cellular functions namely cell fusion, signal transduction, exocytosis, cell differentiation and apoptosis. One of the most studied areas is the cleavage of cytoskeletal attachment. Calpain's roles in this particular function have been demonstrated in studies which involved cell spreading, cell motility, myoblast fusions of skeletal muscles as well as in fibroblast cells derived from calpain-4 knock-out (KO) mice embryos. The majority of the reports imply that several substrates for calpain are the components of focal adhesion complex as well as the proteins which are involved with signalling pathways in cell migration (Carragher and Frame, 2002). These findings draw attention to the involvement of calpain in the cell motility response and progression. The proteolysis of focal adhesion components at both of the leading and rear edge attachment sites may be required in order for the cells to be motile. This action might involve the adhesion component degradation which leads to the rear edge detachment and the restructuring of new contact locations within the pseudopodia that eventually allow the cells to progress (Glading et al., 2002).

1.7.4.1. Cell Spreading Properties

The involvement of calpain in cell spreading has also been investigated in different cell types. However, no consensus exists with regard to calpain's role in coordinating this process. That notwithstanding, one of the established findings is that calpain is involved in the ability of cells to spread. The inhibition of calpain in platelets, T cells, smooth muscle cells and pancreatic β cells have all demonstrated reduced abilities to spread (Franco and

Huttenlocher, 2005). It is thought that the effect may be a result of decreased proteolysis activity on the focal adhesion complex. The complexity in the cell spreading process involves a dynamic rearrangement of adhesion complex driven by integrin activation which would enable the cells to attach and spread. When the calpain inhibitors were added onto spread cells, it resulted in the marked loss of focal adherence and caused the cells to revert back to their normal round shape (Glading et al., 2002). The ability for the cell to spread is thought to be calpain-1 dependent as observed in the overspreading effects of cells with excessive focal adhesion complexes and calpain-1 overexpression. High levels of available β-actin-binding protein molecules called ezrin in the fibroblast cells has led to the suggestion that cytosolic cleavage of this ezrin tail could caused reorganization of the cytoskeleton and eventually allowing the cells to spread.

1.7.4.2. Regulation of Cell Adhesive Complex and Migration Process

The fundamental understanding of the capacity for cells to migrate is dependent on their capability to adhere onto the extracellular matrix. Regulation of the integrin adhesion complex with the extracellular matrix is one of the important functions in cellular migration. This action requires coordinated cellular signalling between the leading and rear edge of the cells to allow movement. The initial contact at the leading edge of the cells acts as an anchor which regulates adhesions complexes and then communicates with the rear edge to release its attachment. However, the interactions and mechanism of actions within the cells is still vaguely understood. The adhesion strength between cells and the extracellular matrix could perhaps have an important role in cell migrations, as the adhesion complexes with greater strength fails to detach when calpain is inhibited and this is contrasting to the cells with low

strength adhesiveness (Perrin and Huttenlocher, 2002). It is suggested that calpain weakens the integrin intracellular binding and its detachment from the cells cytoskeleton structures will subsequently promote cells migration. Inhibiting calpain appears to stabilize the cellular focal adhesions and reduced detachment rates of the cells (Huttenlocher et al., 1997).

Molecules found residing at the focal adhesion complex such as paxillin, talin, ezrin, α-actinin, focal adhesion kinase (FAK) as well as β1, β2 and β3 cytosolic tails provides a large number of potential targets for calpain (Glading et al. 2002). Accordingly, the involvement of calpain following the cellular signalling mechanism depends on the target substrates and its cleaving action on the substrates. One of the main characteristics of calpain actions is the ability to weaken and modify targeted substrates, which as a result reorganize the adhesion complex structures or affects the cells signalling pathways. Large focal adhesion complexes were found at the peripheral location in the calpain inhibited fibroblast cells (Huttenlocher et al., 1997). It is believed that calpain action on the focal adhesion components could have altered the whole structure before allowing the engagement of different proteins to change the cytoskeletal frameworks or destabilizes the structure (Potter et al., 1998). This action may possibly facilitate the attachment-detachment properties of the cells and as a result promotes cell migration.

It has also been proposed that migrating cells require a coordinated mechanism for their focal adhesion component assembly and disassembly at the leading and rear edges of the cells. However, there is still limited understanding on how the leading edge of the cells corresponds with the trailing edge in order to facilitate cells movements. Potentially, several integrin associated proteins such as talin, actin and vinculin have been found localizing at

the leading edge of migrating cells (Cox and Huttenlocher, 1998). It is believed that the rapid regulation of integrin assembly at the leading edge happen simultaneously with disassembly of integrin at the rear edge of the cells which orchestrates the migration process. Regulatory mechanisms for the migrating cells may also involve the influx of Ca^{2+} and calpain-mediated activity (Schaff et al., 2008). The effects of Ca^{2+} influx alone might possibly have different roles in migration but this depends on the type of cells observes. How this system actually works is still unknown, but this could point to the correlation between Ca^{2+} and the Ca^{2+}-activated calpain during the migration process. Calpain is believed to be a major part in the integrin assembly as well as in leukocytes cellular adhesion, and inhibition of calpain in the fibroblast cells reduced its spreading ability (Potter et al., 1998). This suggests that calpain acts as regulatory enzyme for the adhesion complex assembly and disassembly in the cells.

1.8. Neutrophil Regulation by Calpain

1.8.1. Calpain in Neutrophil Adhesion and Spreading

The first crucial step in neutrophil recruitment is to the ability to adhere on surfaces such as endothelial cells or extracellular matrices. Circulating neutrophils are characterized by their spherical shapes with a permanent wrinkled surface. The “unwrinkling” mechanism is associated with the β2-integrin molecules that are found on the cell surface and is said to be correlated to cellular adhesion and spreading (Diacovo et al., 1996). The neutrophil “unwrinkling” may involve cleaving the cross-linkages of binding molecules such as talin and/or ezrin that are attached to the cell cytoskeleton and within the wrinkles themselves, and therefore allowing the cells to spread (Dewitt and Hallett, 2007). It has been well-documented that an increase in cytosolic Ca^{2+} level causes neutrophils to spread. This

system is thought to be related with the Ca^{2+}-dependent calpain actions which release the molecular cross-links between the cells and the extracellular matrix. During the process, activated calpain would sever the bond between the actin cytoskeleton and the plasma membrane to facilitate the membrane expansion of the cells and eventually cell spreading. It has been established that talin is cleaved during physiological Ca^{2+} influx in neutrophils which suggests the involvement of specific calpain proteolytic actions (Sampath et al., 1998). The majority of the earlier studies focusing on calpain actions and its involvement in cell spreading in different types of cells have demonstrated almost similar mechanisms which involves the cleaving of focal adhesions complex (Ivetic and Ridley, 2004). In the case of neutrophils, the presence of potential substrates for calpain within the focal adhesion complex has made it quite difficult to point to a particular substrate that is associated with the cell spreading capability.

One of the main molecular mediators in the inflammatory reaction is tumor necrosis factor alpha (TNF-α) which is released by the cells involved upon inflammatory stimulation (Wiemer et al., 2010). The release of TNF-α will enhance the signals before eventually inducing the neutrophil adhesion process. The signals generated by TNF-α molecules activates the integrin molecules and leads to neutrophil adhesion on the cellular surfaces (Williams and Solomkin, 1999). Calpain inhibition of TNF-α induced neutrophils shows that the cells ability to adhere on fibrinogen coated surfaces is disrupted as well as the impairment of the focal adhesion complex which contains the molecule vinculin (Wiemer et al. 2010). The reports also demonstrated several other effects mediated by calpain inhibition in a TNF-α induced condition in neutrophils, which includes the cells inability to migrate due to the failure of establishing firm adhesion on the cellular surfaces.

1.8.2. Proposed Roles of Calpain in Neutrophil Migration

The neutrophil migration process is a fascinating subject that has been discussed for years. The majority of earlier studies focused on neutrophil signalling and other molecular mediators that regulate the cells before subsequently promoting migration. One of the most important findings is the role of Ca^{2+} in neutrophils as the major factor in largely every bit of the cell's repertoire. The cells dependency on Ca^{2+} influx has raised the possibility of calpain involvement as one of the main enzymatic regulators of neutrophil function. Although generally calpain has been widely reported to be involved in cell adhesion and migration, this matter was not conclusive when it comes to the course of action concerning neutrophil migration. It is believed that migration in rapidly moving cells such as neutrophils requires a concerted mechanism between the leading front edges and the trailing edges of the cells to exhibit what is seen as a directed gliding motion.

Neutrophil detachment from the extracellular surfaces was not affected by calpain inhibition (Perrin and Huttenlocher, 2002). In addition, inhibiting calpain did not weaken the integrin intracellular bonds in neutrophils or leave trace of the detached integrin on the cell's migration surfaces. On the contrary, it is believed that the capability of neutrophils to migrate involves different mechanisms and that the integrin is endocytosed by the cells. This is an interesting finding as reported by Glading et al. (2002) who mentioned that in other cells, chemotaxis was inhibited by inhibiting calpain activity and that integrin could be one of the required molecules in chemotaxis. Talin, α-actinin and vinculin are the proteins that bind integrin to the actin filament cytoskeleton and it has been found that talin co-localized with the F-actin at the leading edge of migrating neutrophils (Lawson and Maxfield, 1995).

However in the intracellular free Ca^{2+}-buffered neutrophils, the clustering talin was instead seen at the rear edge of the cells and became trapped together with the integrin which facilitates cell attachment to the migration surfaces. By gathering this input and looking at the presence of talin which is a part of the cellular focal adhesion complex as well as Ca^{2+} influx effects in migrating neutrophils, it suggests that the mechanism of action is ideal for calpain action. However, previous reports have indicated that this may not be the case and raises further questions on the roles of calpain in the neutrophil migration process, in addition to what makes calpain functioning differently in neutrophils as compared to the other type of cells.

It has been said that neutrophils have poorly constructed integrin clusters associated with the focal adhesion complex. By looking at the rear edges of migrating neutrophils, it has been shown that the integrin families of β1, β2 and β3 integrin are still intact at the cell surface and are believed to be endocytosed after migration (Cox and Huttenlocher, 1998). The incident is said to be related with the integrin "recycling" during the neutrophil migration process. Lawson and Maxfield (1995) reported that the integirn chains of αv and β3 were highly concentrated at the leading front of neutrophils migrating on vitronectin coated surface as compared to the percentages at the rear edge. It showed that Ca^{2+} is required in order to break the formation of αvβ3 integrin with the adhesion complex that is associated with the protein talin at the front edge of the migrating neutrophils. It has been suggested that the αvβ3 integrin at the trailing edges are endocytosed before subsequently being recycled forward to the leading front of the migrating cells. Although this study describes the fate of integrins and Ca^{2+} requirements within the cells, it did not explain the mechanisms that break the integrin clusters before the endocytosis and recycling process.

Based on this information, calpain has been identified as one of the potential regulatory enzymes which might be involved in this event and sever the linkages between the integrin and adhesions complex (Molinari and Carafoli, 1997). In chinese hamster ovary (CHO) cells, calpain inhibition has significantly reduced the amount of the integrin molecules which have been ripped from the cell surfaces during rear detachment and indicated that calpain might facilitate rear edge release (Palecek et al., 1998). In neutrophils, this could be a probable mechanism of action that involves the Ca^{2+} influx within the cells which will activate calpain action on potential substrate such as talin or ezrin, and then destabilizes the whole focal adhesion complex.

The role of calpain in neutrophils has been highlighted in several chemotaxis studies. It was reported that the constitutive activity of calpain may act as a negative regulator for membrane expansion and migration in neutrophils (Franco and Huttenlocher, 2005; Katsube et al., 2008; Lokuta et al., 2003). Furthermore, inhibition of calpain leads to the diminishing effects in neutrophil chemotaxis and the cell's directional senses towards the fMLP and IL-8 (Interleukin-8) concentration gradients. In addition, it has been suggested that the negative regulatory effects on the cells directional migration is mediated by calpain through its action on the activity of Rho family of GTPases (Ras homolog family of guanosine triphosphatases) namely the Cdc42 (Cell division control protein 42 homolog) and Rac (Katsube et al., 2008). In resting neutrophils, calpain-1 is found to be the key active isoform. By inhibiting calpain-1 the polarization and chemokinesis of neutrophils were reported to be stimulated (Lokuta et al. 2003). This event is suggested to be related to a specific inhibitory action on calpain-1 and this enzyme could possibly have a role during the cell migration process.

Katsube et al. (2008) supported the proposed idea of calpain function as a negative regulator in neutrophils by looking at the neutrophil-like HL-60 cells. In addition, it is shown that neutrophils were capable of undergoing chemotaxis after inhibiting calpain and activating the MAPKs (mitogen-activated protein kinases) signalling pathways which promotes active migration. It is believed that the suppression of distinct signalling pathways by calpain help to restrain neutrophil migration and polarization which therefore maintains the spherical shape in resting cells. However, these cellular effects could only be observed in mature neutrophils and specifically when calpain is inhibited in the HL-60 cells at the stage of differentiation into mature neutrophils. At resting state, the level of calpain in neutrophils is high and following inhibition of this enzyme, rapid chemokinesis together with membrane protrusion were observed (Franco and Huttenlocher 2005). Although these reports recorded different findings, both of them agreed that inhibiting calpain activity promotes neutrophil progression to a certain extent. However, both reports did not look into the involvement and presence of Ca^{2+} as the key signalling molecule for neutrophils as well as how Ca^{2+} affected the negative regulatory actions carried out by Ca^{2+}-activated calpain. Furthermore, evidence of a high calpain activity level in resting neutrophils is not thoroughly addressed particularly on the calpain-mediated suppression in maintaining the cell's spherical form.

Neutrophil migration to the inflammation site begins with their ability to arrest and adhere to the endothelial cells. One of the molecules that has been identified and shown to promote the migration process is TNF-α. The adhesion of neutrophils is mediated by the human lymphocyte homing receptors or also known as LAM-1 via TNF-α activated signalling mechanism on human umbilical vein endothelial cells (Spertini et al., 1991). TNF-stimulation in neutrophils is shown to bring the cells to a stop and disrupts its random migration (Smart

and Casale, 1994). It has been demonstrated that in the absence of TNF-α neutrophils were loosely adhered on the surface. By treating neutrophils with TNF-α in addition to a more potent calpain inhibitor, the migration speed of the cells are significantly increased (Wiemer et al., 2010). This suggests that calpain inhibition has blocked circulating neutrophils from coming to a halt and this mechanism is mediated through the TNF-α signalling pathway.

Calpain has also been implicated in neutrophil migration *in vivo* in particular calpain-1. An *in vivo* study showed that neutrophils recruitment to inflammatory sites is markedly reduced after being treated with the calpain-1 inhibitor (Cuzzocrea et al., 2000). Swelling in the hind paws of collagen-induced arthritic rats were measured accordingly and histological assessment proved that the degree of arthritis after treatment with calpain-1 inhibitor was significantly reduced. This finding demonstrated that in the animal model, inhibiting calpain activity leads to less infiltration and accumulation of white blood cells which includes neutrophils. This result may be due to calpain inhibitory effect which hindered recruitment of inflammatory cells, including neutrophils, from migrating to the target site. Nevertheless, the findings from Cuzzocrea et al. (2000) and Wiemer et al. (2010) offered evidence on a very interesting subject and speculative notion with regards to neutrophil migration ability. Prior to migration, neutrophil ability to adhere and spread on to the extracellular matrices such as the endothelial cells is an essential step. Lokuta et al., 2003 reported that calpain inhibition did not cause neutrophils to tether on the migrating surface nor will it restrict the migration speed, but the idea of the cells migrating without having the ability to adhere on to the surface matrices with increased migration speed is rather contradictory. The lack of neutrophil infiltration after calpain inhibition in inflammatory disorders such as arthritis could also indicate the loss of “homing” signals, and as a result caused the cells to migrate in

random. Therefore, this information provides an opportunity to further explore the role played by calpain in neutrophil migration.

Several findings as well as ideas have been proposed on the potential role of calpain in regulating neutrophil function, particularly on their migration capability (Figure 1.8.2.1). This includes a number of probable mechanisms that follows the inhibitory effect on calpain activity, which as has been previously reported showed to be very interesting but with contrasting results. By looking specifically into the participation of Ca^{2+} as the key molecule which is involved in most neutrophil functions and its interaction with the enzyme calpain, this would provide a better notion on this matter. Establishing this interaction could possibly be the major point in trying to understand how calpain would regulate the migration process in neutrophils or perhaps cell functions as a whole.

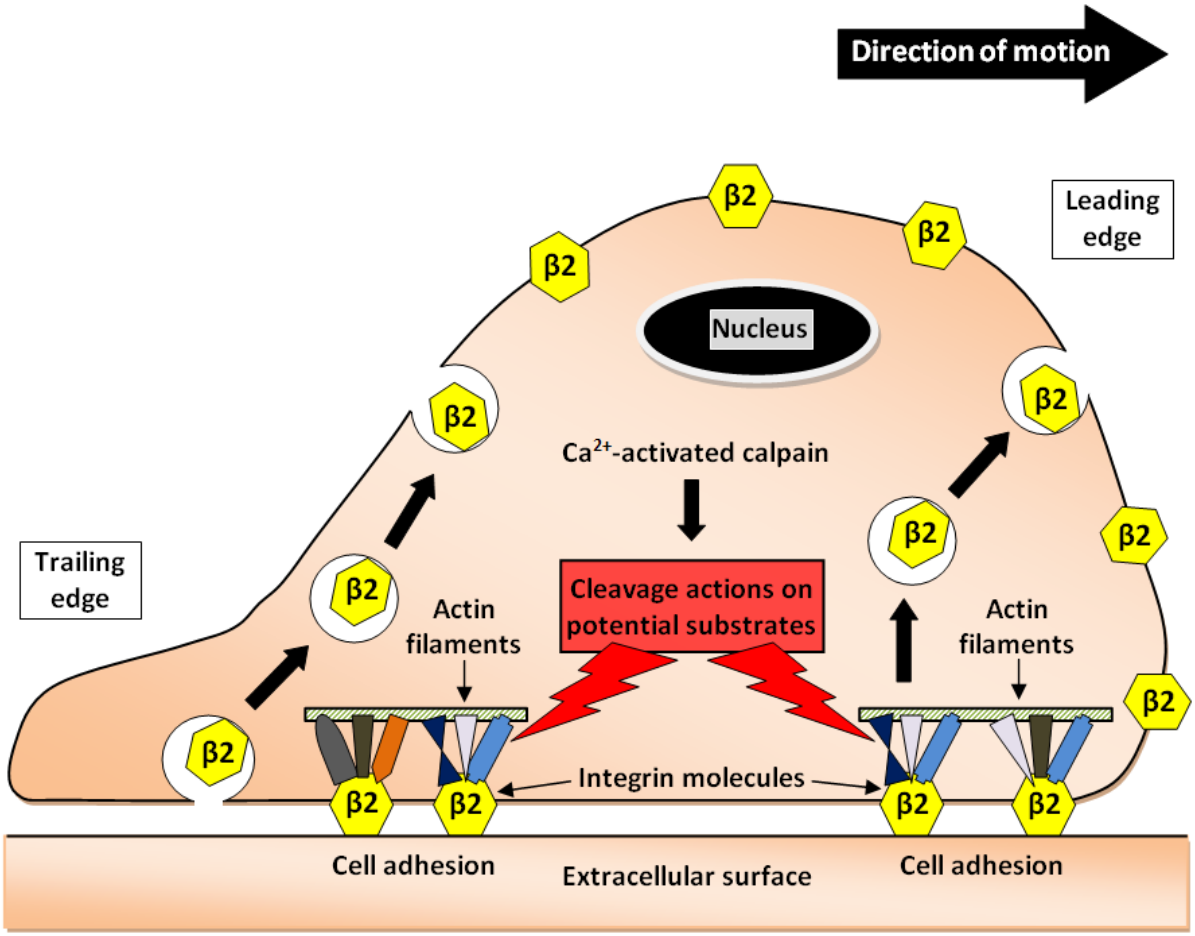


Figure 1.8.2.1: Possible mechanism of action for neutrophil migration. This figure based on the previous reports illustrates calpain cleavage actions on its potential substrates with the β2-integrin molecules recycling process at the front and rear of migrating neutrophils.

1.8.3. The Roles of Calpain in Phagocytosis

Neutrophils are well-known for their ability to phagocytose any foreign or invading microorganism. As the immune system's first line of defence, the phagocytosis process is one of the several important tasks performed by neutrophils in order to protect the hosts from infection or illness. Phagocytosis function is enhanced after neutrophils make contact and becomes activated by C3bi-opsonized particles (Berger et al., 1984). The consequential event from this mechanism is achieved through the interaction between the C3bi-opsonized particle and one of the integrin family members, namely the β2-integrin (Figure 1.8.3.1). Neutrophils mainly express β2-integrin on their membrane surface (Lawson and Maxfield, 1995). It has also been shown that the β2-integrin is inactive in non-activated neutrophils. In resting neutrophils, it appears that only a small fraction of β2-integrin are detected (Dewitt et al., 2002), and the influx of cytosolic free Ca^{2+} within the cells significantly increases the mobility of β2-integrin. It is suggested that this action is coupled with the presence of Ca^{2+}-activated calpain activity (Figure 1.8.3.1). The calpain enzyme is believed to be involved in the untethering of β2-integrin from the cellular cytoskeleton bonds. By treating neutrophils with calpain inhibitor and then increasing their level of cytosolic free Ca^{2+}, the mobility of β2-integrin has been dramatically decreased to a level lower than in the cells at resting state (Dewitt and Hallett 2002).

The β2-integrin is associated with the phagocytosis process, and this action normally progresses after establishing the cross-link between neutrophils with the opsonized particle before forming the phagocytic "cup" and then engulfing it. Neutrophils treated with calpain inhibitors and presented with opsonized particles demonstrated that the β2-integrin is not

available in the phagocytic cup although the molecule is evenly distributed on the surface of the cells (Dewitt and Hallett 2002). The calpain inhibitor treated cells still displayed their capability to phagocytose but with slower reaction as well as decreased proficiency to complete the process. By looking at individual neutrophils after calpain inhibitor treatment, some of the cells appeared to be able to bind and form the phagocytic "cup" at the point of contact with the opsonized particles but failed to complete phagocytosis despite showing their Ca^{2+} signalling response. These data when taken together suggested the interactions which involve the β2-integrin molecules, calpain and Ca^{2+} within the cells during phagocytosis. It indicates that calpain is activated following the increase of cytosolic free Ca^{2+} level, and as a result untether the β2-integrin molecules which then allow neutrophils to complete phagocytosis of the opsonized particles. Sampath et al. (1998) proposed that in the non-activated PMNs, β2-integrin molecules are tethered to the cell cytoskeleton via the protein talin. As one of the potential substrates for calpain, releasing the β2-integrin tether may possibly be attributable to the calpain actions on talin and consequently promotes phagocytosis.

Tian et al. (2004) also suggested that the β2-integrin molecules on neutrophils are at stationary positions because the cell cytoskeleton bindings via the protein talin. After talin is cleaved by calpain, the β2-integrin molecules are released and generate signals that allow the cells to move, extravasate and perform phagocytosis. It has also been shown that neutrophils are prevented from completing trans-endothelial migration and phagocytosis of opsonised particles by inhibiting calpain activity (Tian et al., 2004). In addition, these effects are believed to be related to the physiological level of Ca^{2+} in neutrophils which is not high enough for calpain activation. The rapid increase in Ca^{2+} concentration may be restricted to

the location just beneath the plasma membrane and especially in the wrinkles (Brasen et al., 2010), and is not outspread into the cell (Davies and Hallett, 1998). Given the presence of rapid influx of Ca^{2+} within the cells, this could explain the activity of Ca^{2+}-activated calpain and other signalling mechanisms which may occur at the sub-plasma membrane location. With the β2-integrin molecule mainly bound by talin to the extracellular matrix and residing on the cell surface, the activated calpain might act on talin and liberate cell movement. By putting this data together, it gives a clue on the reason why calpain activity is restricted to the plasma membrane sites only and its action during phagocytosis.

In general, most of the previous studies reported a correlation of the calpain activity within neutrophils with either one or more component that is involved in the phagocytosis process. As reported in the cell spreading and migration capability, calpain actions during phagocytosis followed a similar action which basically involves Ca^{2+} influx and restructuring the β2-integrin molecules by cleaving its potential substrates such as talin. It is believed that in phagocytosis, the initial contact between the cells and opsonised-particles is an important step which leads to the substrates to be cleaved by calpain and breaking the β2-integrin link. This will then liberate the cell membrane reservoir, permitting neutrophils to engulf the particles before eventually completing phagocytosis. The data shown in previous reports is very intriguing and provides a good fundamental understanding on neutrophil function as well as their signalling response. However, the regulation of neutrophil functions involving calpain and its action has not been explicitly addressed. It is still rather ambiguous whether neutrophils ability during phagocytosis is due to calpain action on its substrates, particularly talin and ezrin. Thus, this provides a platform for further investigations and determining this mechanism of action may be an important aspect in phagocytosis.

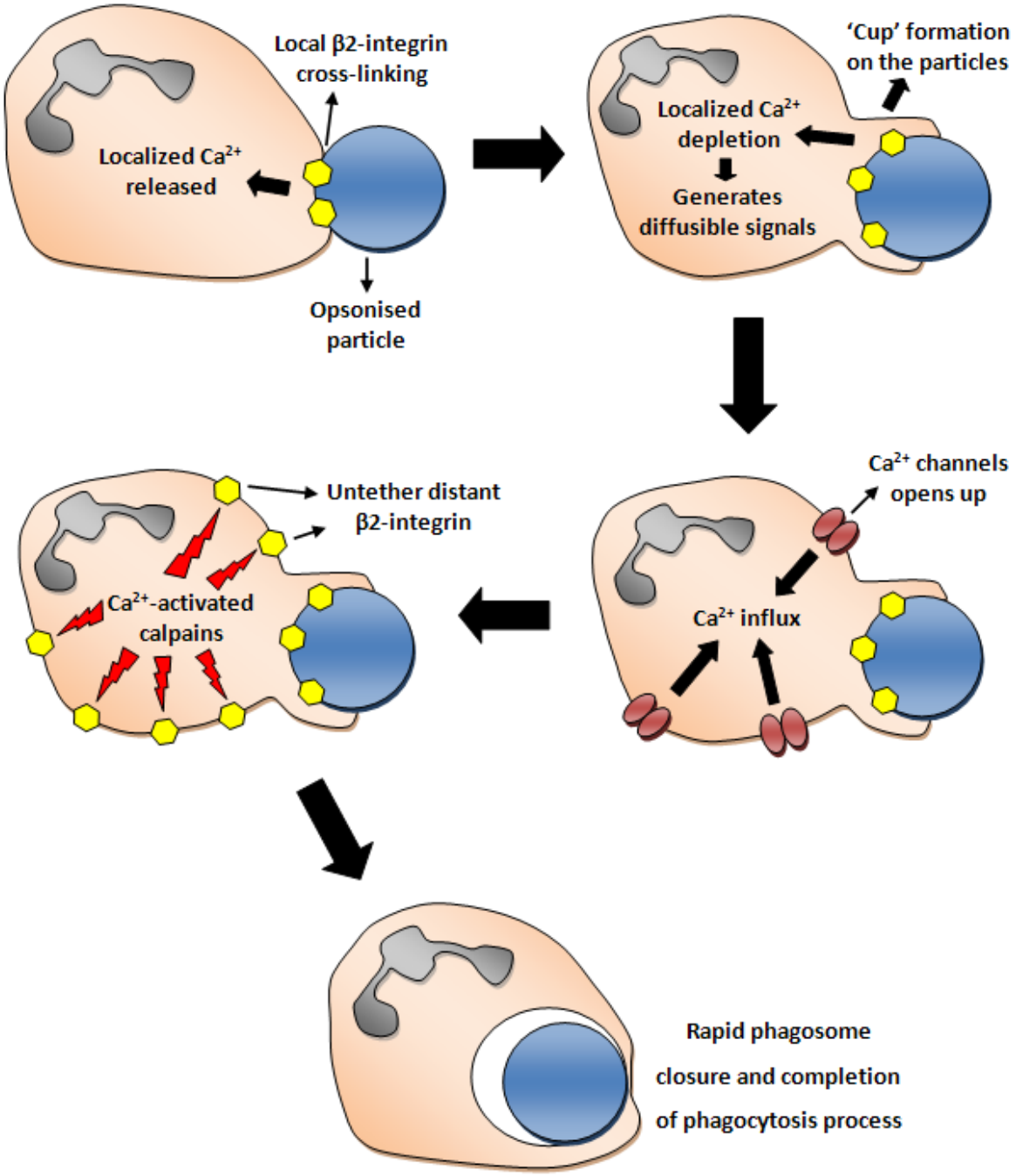


Figure 1.8.3.1: Proposed model of neutrophil reaction towards the opsonised particle. This figure explains the Ca^{2+} role and influx , together with the possible function of Ca^{2+}-activated calpain which leads to the completion of phagocytosis in neutrophils.

1.8.4. Possible Physiological Functions of the Calpain System in Neutrophils

In general, through years of studies the calpain system has been shown to play a part in cell functions which contributed to a number of cell characteristics and it is believed that this system has a major effect on almost every key step from neutrophil ability to adherence and spreading, as well as in the migration and phagocytosis process. It is understood that the calpain system facilitates the ability of circulating neutrophils to adhere onto the blood vessels endothelial cell lining, which then allows neutrophils to spread and transmigrate.

By looking at the enzyme-substrate model, calpain has been associated with several substrates that mainly associate with the focal adhesion clusters of the actin cytoskeleton. Although talin is lauded as the most prominent substrate for calpain, it is yet to be concluded whether calpain's cleavage action on talin alone is the reason neutrophils are able to spread, migrate or phagocytose, or whether these functions are attributable to the calpain activities in the cells. This raises suggestions on the involvement of other substrates which are found in neutrophils such as ezrin/radixin/moesin (ERM) proteins (Ivetic and Ridley, 2004) and kindlin (Moser et al., 2009). Nevertheless, it gives a very strong indication about the effects of calpain and how it might be regulating neutrophil functions altogether. Therefore, determining and understanding the significance of calpain system in neutrophil's is an important subject if this enzyme is to be considered as a potential therapeutic target for illnesses related with inflammatory disorders.

1.9. Aims of the Study

Great interest is put on calpain in general, with numerous proposals and suggestions have been made on how this enzyme affects neutrophil. But, whereas some of the earlier works did not address the influence of Ca^{2+} on calpain activities, some others did acknowledges the importance that Ca^{2+} has on this Ca^{2+}-activated enzyme. There is however uncertainty still revolving on the issues concerning calpain inhibitory effects on the ability of neutrophils to adhere and migrate. Some believed that migration involves calpain cleavage actions on one or more substrates, linking β2-integrin molecules with the actin cytoskeleton while others disagreed. Some reports even suggested that cells are not well adhered on the extracellular surfaces and their migration ability is enhanced after calpain inhibitions. The reports appeared to be conflicting with one another, and thus far no consensus on the role of calpain has been reached.

The working hypothesis of this thesis is that knocking-out calpain-1 prevents neutrophils from performing their normal functions.

The objective of this study is to understand the role of calpain-1 in neutrophil activity and how it regulates the overall functions of the cells. This objective will be achieved using neutrophils isolated from experimental animal models of wild-type and calpain-1 knock-out (KO) mouse. The specific aims of this research are:

1. To generate a colony of normal wild-type and homozygous calpain-1 KO mouse.
2. To determine calpain-1 expression in the normal wild-type and homozygous calpain-1 KO cells.

3. To distinguish the morphological differences between the cells from normal-wild type and homozygous calpain-1 KO mouse.
4. To record and measure the adhesion and spreading capability of normal wild-type and homozygous calpain-1 KO neutrophils in response to fMLP as chemoattractant.
5. To assess the normal wild-type and homozygous calpain-1 KO neutrophil's capability to signal Ca^{2+} via the IP_3 pathways and their reactions.
6. To determine neutrophil *in vivo* recruitment following zymosan injection and *in vitro* trans-endothelial migration following fMLP gradient of the normal wild-type and homozygous calpain-1 KO mouse.
7. To measure and analyse chemotaxis progression in normal wild-type and homozygous calpain-1 KO neutrophils.
8. To examine the ability of normal wild-type and homozygous calpain-1 KO neutrophils to complete phagocytosis process and together with their Ca^{2+} signalling.

Chapter 2

Materials and Methods

2.1. Materials

The reagents, equipment and software programming presented here described the general materials used throughout the entire experimental works.

2.1.1. Reagents

Reagents	**Manufacturers**
Heparin	CP Pharmaceuticals Ltd, U.K.
Fura-2 AM	Invitrogen Ltd, Paisley, U.K.
Fluo-4 AM	Invitrogen Ltd, Paisley, U.K.
IP_3 (Caged)	Enzo Life Sciences, Exeter, U.K.
Mouse Anti-Calpain-1 Primary Antibody (D-11)	Santa Cruz Biotechnology, Germany
Mouse Anti-Calpain-2 Primary Antibody (E-10)	Santa Cruz Biotechnology, Germany
Anti-Mouse IgG-HRP Secondary Antibody	Santa Cruz Biotechnology, Germany
Anti-Mouse IgG-FITC Secondary Antibody	Santa Cruz Biotechnology, Germany
EGTA	Sigma-Aldrich Ltd, Dorset, U.K.
EDTA	Sigma-Aldrich Ltd, Dorset, U.K.
Fibronectin	Sigma-Aldrich Ltd, Dorset, U.K.
Zymosan A	Sigma-Aldrich Ltd, Dorset, U.K.
fMLP (N-Formyl-Met-Leu-Phe)	Sigma-Aldrich Ltd, Dorset, U.K.
Acridine Orange	Sigma-Aldrich Ltd, Dorset, U.K.
Bovine Serum Albumin (BSA)	Sigma-Aldrich Ltd, Dorset, U.K.

Dimethyl Sulphoxide (DMSO)	Sigma-Aldrich Ltd, Dorset, U.K.
Chloroform	Sigma-Aldrich Ltd, Dorset, U.K.
$NaHC0_3$	Fisher Scientific, Leicester, U.K.
Na_2CO_3	BDH Laboratory Supplies, Poole, U.K.
NaCl	Sigma-Aldrich Ltd, Dorset, U.K.
$MgCl_2$	Sigma-Aldrich Ltd, Dorset, U.K.
HCl	Sigma-Aldrich Ltd, Dorset, U.K.
KCl	Sigma-Aldrich Ltd, Dorset, U.K.
Na_2HPO_4	Sigma-Aldrich Ltd, Dorset, U.K.
KH_2PO_4	Sigma-Aldrich Ltd, Dorset, U.K.
$MgSO_4$	Sigma-Aldrich Ltd, Dorset, U.K.
$CaCl_2$	Sigma-Aldrich Ltd, Dorset, U.K.
NaOH	Sigma-Aldrich Ltd, Dorset, U.K.
Na_3VO_4	Sigma-Aldrich Ltd, Dorset, U.K.
Lucifer Yellow	Sigma-Aldrich Ltd, Dorset, U.K.
Glycerol	Sigma-Aldrich Ltd, Dorset, U.K.
Horse Serum Liquid	Sigma-Aldrich Ltd, Dorset, U.K.
V450- LY-6G and LY-6C (Gr-1) Antibody	BD Biosciences, Oxford, U.K.
F4/80 Antigen PE Antibody	eBioscience Ltd, Hatfield, U.K.
Anti-Neutrophil [7/4] (FITC) Antibody	Abcam Plc, Cambridge, U.K.
Tris Base White Crystals Powder	Fisher Scientific, Leicester, U.K.
Glycine	Fisher Scientific, Leicester, U.K.
Sodium Dodecyl Sulfate (SDS)	Fisher Scientific, Leicester, U.K.

Triton™ X-100	Sigma-Aldrich Ltd, Dorset, U.K.
Tween® 20	Sigma-Aldrich Ltd, Dorset, U.K.
Hepes	Fisher Scientific, Leicester, U.K.
Methanol	Fisher Scientific, Leicester, U.K.
NuPAGE® Antioxidant	Life Technologies Ltd, Paisley, U.K.
NuPAGE® Sample Reducing Agent (10X)	Life Technologies Ltd, Paisley, U.K.
SuperSignal West Pico Chemiluminescent	Fisher Scientific, Leicester, U.K.
Glutaryldehyde Solution	Sigma-Aldrich Ltd, Dorset, U.K.
Formaldehyde Solution	Sigma-Aldrich Ltd, Dorset, U.K.
Phosphate Buffer (pH 6.8) tablets	BDH Laboratory Supplies, Poole, U.K.
DPX Mounting Medium	Sigma-Aldrich Ltd, Dorset, U.K.
Hemacolor® Rapid Staining	Merck Chemicals Ltd, U.K.

2.1.2. Equipment

Equipment	**Manufacturers**
Harrier 15/80 Centrifuge	MSE (UK) Ltd, London, U.K.
Microfuge® 18 Microcentrifuge	Beckman Coulter Ltd, U.K.
Cellometer® Automated Cell Counter	Peqab Ltd., Fareham, U.K.
InoLab® pH 720	WTW GmbH, Germany
NewClassic MS Analytical Balance	Mettler-Toledo Ltd, Leicester, U.K.
WPI Micromanipulator	WPI, Florida U.S.A.
Eppendorf Femtojet Pressure Controller	Eppendorf, Hamberg, Germany

Eppendorf 5629 Micromanipulator	Eppendorf, Hamberg, Germany
Micropipette Puller P2000	Sutter Instruments, USA
Micropipette Capillaries	Sutter Instruments, USA
Nikon Eclipse Inverted Microscope	Nikon, U.K.
IC100 Intensified CCD Camera	PTI, Surbiton, U.K.
Rapid Monochromator	Delta RAM, PTI, Surbiton, U.K.
Red Sensitive Camera	Watec, Japan
Filters, Dichroic Mirrors	Omega Optical Inc, Stanmore, U.K.
Beam Splitter	Cairn Research Instruments, U.K.
Microscope Stage Heater	Linkam Scientific Instruments, U.K.
LPS-220b Lamp Power Supply	PTI, U.K.
CLSM Confocal Microscope	Leica, Milton Keynes, U.K.
Dissection Kit	VWR International Ltd, U.K.
IKA T-10 Basic Homogenizer	Cole-Parmer Instrument Co. Ltd, U.K.
XCell II™ Blot Module	Invitrogen Ltd, Paisley, U.K.
UVIprochemi Imaging System	UVItec Ltd, U.K.
Flow Cytometry CyAn™ ADP Analyzer	Beckman Coulter Ltd, U.K.

2.1.3. Software

Software Programs	**Manufacturers**
Microsoft Excel 2000	Microsoft Corporation
Microsoft PhotoEditor 3.0	Microsoft Corporation

Microsoft Word 2007	Microsoft Corporation
Microsoft Paint Version 5.1	Microsoft Corporation
Paintshop Pro Version 4.15 SE	Jacs Software Inc., USA
Adobe Photoshop 7.0	Adobe Systems Inc, USA
Image J	National Institute of Health, USA
Gif Animator (32-bit)	Alchemy Mindworks Inc., Canada
Image Master 1.4b8	PTI, U.K.
EasyRatio Pro	PTI, U.K.
Ulead Video Studio	Corel UK Ltd, Berks, U.K.
HyperCam Version 1.34.00	Hyperionics Technology, USA
UVIsoft for UVIprochemi Imaging	UVItec Ltd, U.K.
HyperCyt Software Interface CyAn	Beckman Coulter (U.K.) Ltd

2.2. Buffers

2.2.1. Hepes Buffered Krebs Medium

The Hepes Buffered Krebs (HBK) medium: 120mM NaCl; 25mM Hepes; 4.8mM KCl; 1.2mM KH_2PO_4; 1.2mM $MgSO_4.7H_20$; 1.3mM $CaCl_2.2H_20$ and 0.1% (w/v) of BSA.

The HBK stock solutions were prepared in double distilled water. Both NaCl and Hepes stock solutions were stored at 4°C. KCl, KH_2PO_4, $MgSO_4.7H_20$ and $CaCl_2.2H_20$ were dispensed into aliquot (20ml) which was kept at 4°C for immediate usage or frozen for future use. Fresh HBK medium was made from these stock solutions for every experiment with the addition of BSA to a final concentration of 0.1% (w/v) (stock BSA was prepared by

diluting 10% (w/v) in ddH_20 and frozen in aliquots of 1ml). HBK was then adjusted to pH 7.4 using NaOH.

2.2.2. Balanced Salt Solution

The Balanced Salt Solution (BSS) contain: 130mM NaCl; 2.6mM KCl; 8.0mM Na_2HPO_4 and 1.83mM KH_2PO_4. Batches of 5 litres BSS were made by dissolving 40g NaCl, 1g KCl, 5.75g Na_2HPO_4 and 1g KH_2PO_4 in double distilled H_2O and adjusting to pH 7.4 using NaOH.

2.3. General Methods

This section describes the general methodology used throughout this research work.

2.3.1. Blood Collection and Neutrophil Isolation Protocol

Blood samples were collected from the mice through cardiac puncture in accordance to the Schedule 1 procedures of the Animals (Scientific Procedures) Act 1986. Neutrophils were isolated from heparinised blood of the mice as described in these following steps:

1. Heparinised blood (10μl heparin/1ml blood) was added to BSS (25ml) and centrifuged at 350xG for 2 minutes.
2. The supernatant was discarded and the red blood cells were removed by hypotonic lysis, by resuspending the cells in double distilled water (about 1-2ml) for 30 seconds.
3. The cells were restored to osmolarity by diluting them with BSS at pH 7.4 (25ml) and then centrifuged at 350xG for 2 minutes.

4. The supernatant was removed and the cells were suspended in HBK medium with BSA (1ml). The cells were kept on ice until further use.

2.3.2. fMLP Solution

The fMLP powder was weighed (1mg) and was diluted in dry DMSO (2.3ml) to make 1mM stock solution. For experimental purposes, the fMLP stock solution (1µl) was diluted in HBK medium and four to five drops were added to the cells accordingly.

2.3.3. Loading Neutrophils with Fura-2 or Fluo-4 Ca^{2+} Indicators

Fura-2 and Fluo-4 are fluorescent Ca^{2+} indicators which are normally used to detect and measure Ca^{2+} changes in cells. They were loaded into neutrophils in a lipophilic state, as Fura-2 AM and Fluo-4 AM (acetoxymethyl) esters. Once Fura-2 AM and Fluo-4 AM were inside the cells, the intracellular esterases digest these ester bonds and leaving hydrophilic fluorescent Fura-2 and Fluo-4 trapped within the cell (Figure 2.3.3.1). Both Fura-2 AM and Fluo-4 AM were prepared with the same method. Fura-2 AM and Fluo-4 AM were weighed (50µg) and diluted in dry anhydrous DMSO (10µl) before being combined with pluronic F127 DMSO (5µl) in order to facilitate uptake of the dye. The Fura-2 AM and Fluo-4 AM stocks were stored at -20°C until it was required. Prior to the experimental works, Fura-2 AM or Fluo-4 AM (1µl) was added to neutrophils in suspension (1ml) for about 45 minutes at room temperature to allow the cells to take up the dye. The cells were then kept on ice until use.

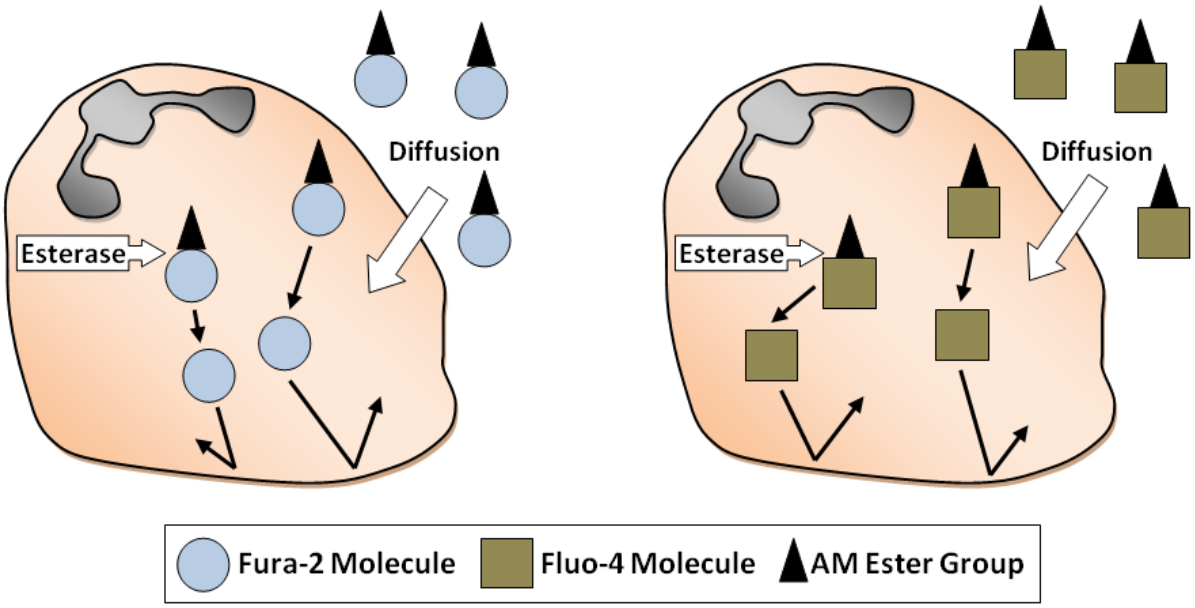


Figure 2.3.3.1: Fura-2 and Fluo-4 acetotoxymethyl (AM) Ca^{2+} signal detection. Fura-2 and Fluo-4 were initially loaded into neutrophils in their acetoxymethyl (AM) ester form of Fura2-AM and Fluo-4-AM respectively. The non-specific esterases in the neutrophil cytosol will then digest the ester bonds and leave fluorescent Fura-2 and Fluo-4 Ca^{2+} indicators trapped within the cells.

2.3.4. Cytosolic Free Ca^{2+} Measurement in Neutrophils

In order to measure the Ca^{2+} signals using either Fura-2 or Fluo-4, neutrophils loaded with the indicators were left to adhere onto glass coverslips maintained at 37°C using the temperature controlled stage heater for about 5-8 minutes. The coverslip was then washed twice with HBK (BSA) solution to remove unbound cells, debris and excess Fura-2 AM or Fluo-4 AM in the solution. After washing, approximately 100μl HBK was returned to the coverslip.

Fura-2 is a Ca^{2+} indicator that displays a marked change in its fluorescent excitation spectrum when it binds to Ca^{2+}. Both of the bound and unbound Ca^{2+} forms of the Fura-2 are fluorescent, and generally two wavelengths of 340nm and 380nm were selected on either side of the emission points representing the two different Ca^{2+} binding states of the probe. When Fura-2 is unbound, the fluorescence excitation is at maximum at 380nm wavelength. When bound to Ca^{2+}, the spectrum shifted making the 380nm signal decrease whereas the fluorescence excited at 340nm increases (Figure 2.3.4.1). In both Fura-2 states, emission wavelength is at 512nm and the ratiometric fluorimetry uses these properties of Fura-2 to quantify changes in the cytosolic concentration of free Ca^{2+}. When the free Ca^{2+} concentration increases in the cells, the amount of unbound Fura-2 decreases as it binds to the free Ca^{2+}. Computer software is programmed to calculate the ratio between these bound (340nm) and unbound (380nm) Fura-2. The Fluo-4 also detects the increase of free Ca^{2+} concentration that is fluorescently excited but using argon-ion laser and without the spectral shift. Fluo-4 is the improved version of the previous Fluo-3, with the distinct improvement of having a brighter fluorescence emission due to greater absorption at

488nm (Figure 2.3.4.1). Using the designated computer software, the increase in free Ca^{2+} concentration in the cells would then bind to Fluo-4 and give brighter fluorescence intensity for measurement. With a more efficient fluorescence excitation than the Fluo-3, lower intracellular concentrations of Fluo-4 can be used to generate the same signal intensity.

The cells were viewed under a selected objective of an inverted confocal microscope and excitation wavelengths of 340nm and 380nm for Fura-2, or 488nm for Fluo-4 were selected. Images at each excitation wavelength were collected using a camera that was connected to the inverted microscope and ratio images were calculated using the respective microscope software. Ratio Ca^{2+} pictures with Fura-2 were recorded and pseudo-coloured accordingly, and the average ratio value of the pixels was calculated before being plotted over the time course. The stimulus was added to the cells under observation while recording continuously. By using Fluo-4, Ca^{2+} signals were detected by the increase in its fluorescence intensity as the images were recorded over a selected time period. Accordingly, the images of fluorescence intensity changes within the cells were measured and plotted into graphical representation.

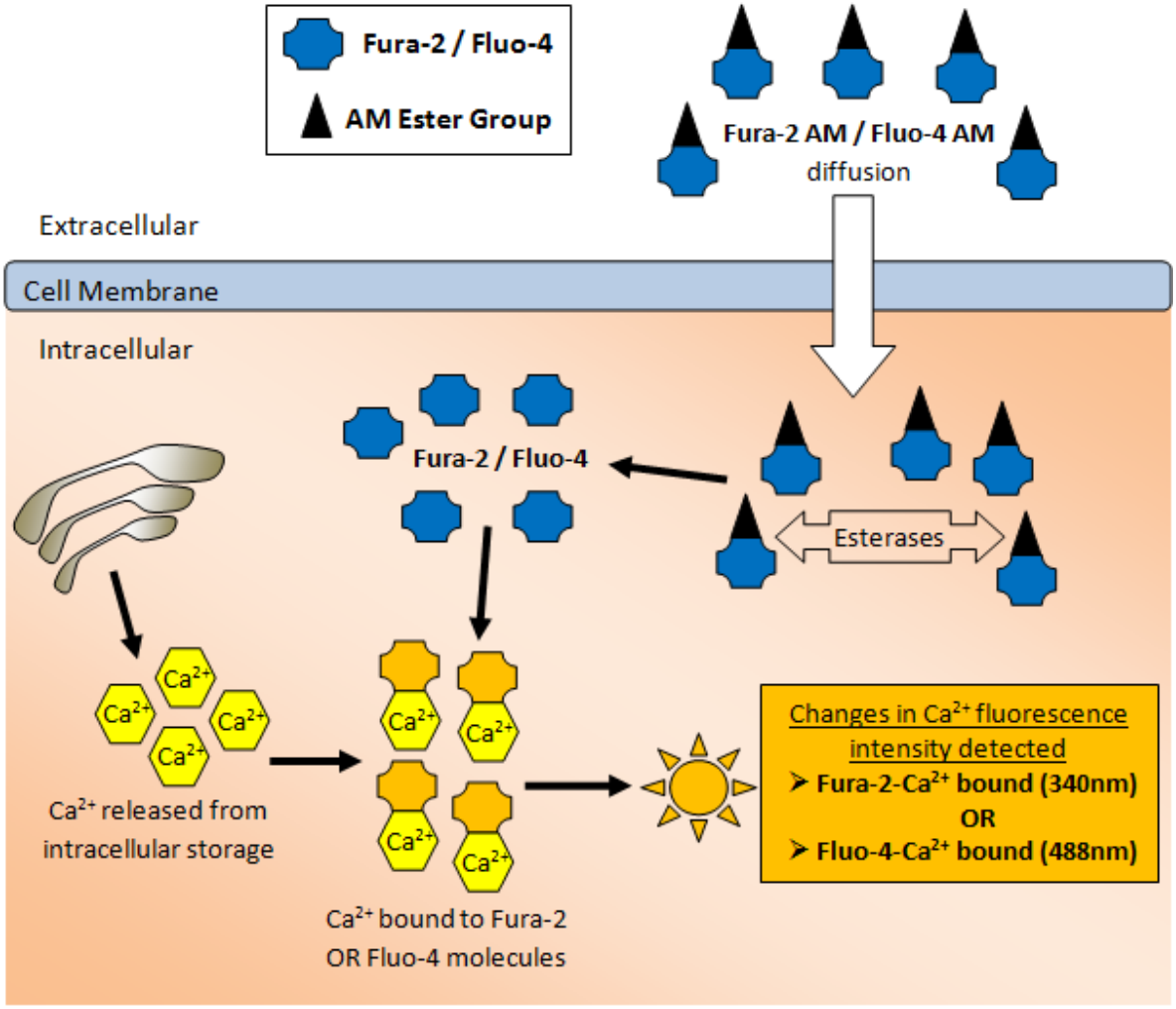


Figure 2.3.4.1: Intracellular Ca^{2+} signal of Fura-2-Ca^{2+} or Fluo-4-Ca^{2+} bound molecule. The diagram describes the rising intracellular Ca^{2+} concentration which resulted in the increase of the Fura-2-Ca^{2+} or Fluo-4-Ca^{2+} bound molecule numbers, respectively. This leads to a change in the Ca^{2+} fluorescence intensity detected and is measured at 505nm wavelength for Fura-2 or 516nm wavelength for Fluo-4.

Chapter 3

Generation of Calpain-1 Knock-out Mouse Colony

3.1. Introduction

This chapter explains the generation of a calpain-1 KO mouse colony in order to raise mice of different genotypes, in particular the normal wild-type and homozygous mice. The work presented here describes the phenotypic and genotypic identification of the colony.

3.1.1. Calpain-1 Knock-out (KO) Mouse

The generation of a calpain-1 KO mouse colony is important for this research as this will be the source of isolated neutrophils for every experiment. One of the most important elements in obtaining reliable results is to maintain a healthy colony of the calpain-1 KO mice. Calpain has been implicated in cellular functions such as apoptosis, cell motility, reorganization of cytoskeleton and cell proliferation in a normal physiological system. It is possible that deleting the gene that produces calpain-1 will subsequently caused disruption to these cellular responses. The first colony of calpain-1 KO mice were generated using the homologous recombination techniques in embryonic stem cells to inactivate the gene that produced calpain-1 (Azam et al., 2001). Generally, the calpain-1 deficient mice were healthy. However, although calpain-1 deficient mice had a normal bleeding time, the clot retraction and platelet aggregation in this animal was significantly reduced. The mice also appeared to be fertile and viable.

The heterodimeric structures of calpain raised the possibility that calpain-1 absence may have an effect on the stability of its smaller subunit calpain-4, and this could affect the sustainability of the mice colony for the long term. The homozygous calpain-4 KO embryos

died during midgestation due to defects to their cardiovascular system, the accumulation of erythroid progenitors and bleeding (Arthur et al., 2000; Zimmerman et al., 2000). The same fate was also reported in calpain-2 KO embryo. The embryos in the calpain-2 KO mice died prior to the implantation stage and suggested that the calpain-2 enzyme is essential during the early embryogenesis period (Dutt et al., 2006). Therefore, the effects demonstrate that embryonic growth depends on the presence of calpain-4 gene and led to the conclusion that one of either the catalytic subunit of calpain-1 or calpain-2 has distinct physiological roles for normal development at the early stages.

3.2. Aims of the Chapter

The aims of the works described in this chapter were therefore to:

1. Set up a breeding plan in order to establish a colony of healthy normal wild-type and homozygous calpain-1 KO mice, and at the same time observing their viability.
2. Identify phenotypic differences between normal wild-type and homozygous calpain-1 KO mice and to confirm their differences by genotyping.
3. Determine calpain-1 expression in the tissue samples taken from the normal wild-type and homozygous calpain-1 KO mouse.

3.3. Methods

3.3.1. Generating Calpain-1 KO Mouse Colony

The novel calpain-1 KO mouse sperm were obtained from the Azam et al. (2001) group which is based in Tufts University School of Medicine, Boston, America and are stored

at the European Mutant Mouse Archive (EMMA) in Oxford, UK. The first colony of the heterozygous calpain-1 KO mouse was generated at the EMMA using intracytoplasmic sperm injection (ICSI) procedure. Sperm of calpain-1 deleted gene was injected into an egg from the C57BL/6 strain mouse. Through this technique, the heterozygous mice were successfully generated and two males and five females were sent to Cardiff University. The mice were kept in sterile isolator cages to acclimatize and paired as breeders. The pups produced from these heterozygous breeders were inspected for any physical defect, and their viability was observed. The pups were genotyped to confirm their genotypic status and paired accordingly to generate the homozygous and normal wild-type mice. The pups generated from homozygous breeders were monitored for physical deformity and viability.

3.3.2. Phenotype and Genotype of Wild-type and Calpain-1 KO Mice

The normal wild-type, homozygous and heterozygous mice were closely examined for abnormalities in their physical appearances including size, body shape and fur colour. The mice were then genotyped for confirmation by taking about 2mm of ear tissues using a puncher. Lysis of the ear tissues was achieved using the DirectPCR® Lysis Reagent (Peqlab Biotechnologie GmbH). The solution was prepared by adding fresh Proteinase K (15µl) into DirectPCR® Lysis reagent (1.5ml) before the solution (120µl) was added to the ear tissues. The ear tissues were put in a hybridization oven at 55°C for 3-16 hours to complete the lysis process. The crude lysates were then incubated in a water bath for 45 minutes at 85°C and centrifuged at 350xG for 10 seconds. For the genotyping protocol, three primers (Sigma-Aldrich) were used; this consists of the two endogenous gene specific primers, GS[E] for

endogenous allele and GS[E,T] for both endogenous and targeted allele, together with one multiplex targeting vector primer NEO[T] for targeted allele.

The gene sequences for the primers were as follows:

GS[E] oligonucleotide sequence: CCATGAAAACGCCATCAAGTACCTG

GS[E,T] oligonucleotide sequence: CCCAGTGCTCCCTGGCAGATGTCTG

NEO[T] oligonucleotide sequence: GGGCCAGCTCATTCCTCCCACTCAT

100µM of these stock primers were diluted to 10µM for the PCR procedure.

Then, PCR composition (19.7µl) was prepared by adding PCR Master Mix (NEB, UK) (10µl) with sterile distilled water (6/7µl) and the ear tissues (0.7µl) in two sets of PCR tubes. The first set (set A) contains all three primers for the targeting vector and both of the endogenous primers (1µl); and the second set (set B) contains only the two endogenous primers (1µl) (Figure 3.3.2.1). The PCR machine was programmed for 35 cycles starting with, 30 seconds of the denaturing process at 95°C, 30 seconds of the annealing process at 60°C and the extension steps for 40 seconds at 68°C. After that, the sample underwent the electrophoresis process in 2% (w/v) agarose gels at 100 volt for 45 minutes. The DNA bands were stained by immersing the gels in diluted ethidium bromide and photographed. The genotyping results were interpreted accordingly.

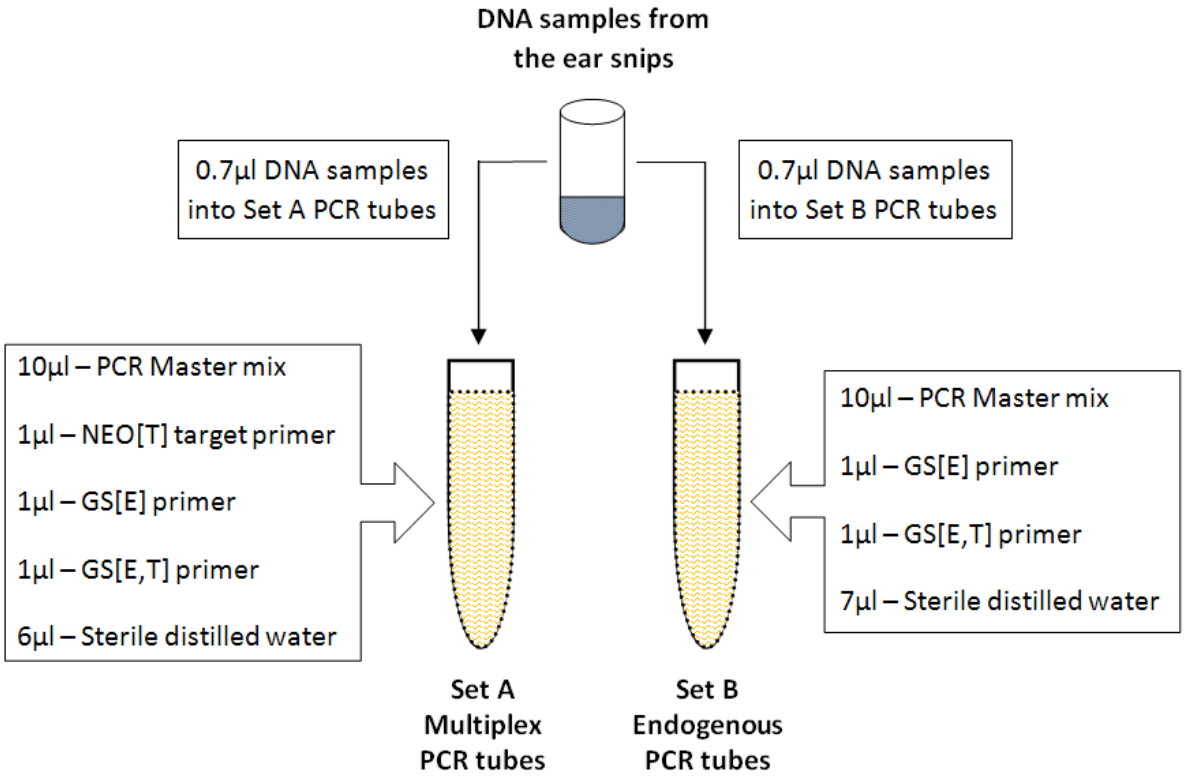


Figure 3.3.2.1: The PCR composition for genotyping using the mouse DNA samples. This figure explains the PCR composition for genotyping using the mice DNA samples from their ear tissues. The two sets of PCR reaction solutions (set A and set B) were run side by side during the gel electrophoresis process.

3.3.3. Detecting Calpain-1 Expression in the Mice Tissue Samples

The calpain-1 expression is identified using the western blot technique. Prior to this work, the lysis buffers for tissue samples were prepared accordingly. The lysis buffer (20ml) contains: 0.5% (w/v) SDS; 1% (v/v) Triton X-100; 10% (w/v) Glycerol; 50mM Hepes; 150mM NaCl; 1.5mM $MgCl_2$; 5mM EGTA; 5mM EDTA, 1mM Na_3VO_4 and topped up to 20ml with distilled water.

The normal wild-type and homozygous calpain-1 KO mice were culled following the Schedule 1 procedure and the liver, bone marrow and red blood cells (RBC) were harvested. The tissue samples were prepared by weighing it and adding the lysis buffer solution (three times the weight of the samples). Then, 1 in 100 volume of the protease inhibitor cocktail (Sigma Aldrich) was added to preserve the proteins in the tissue samples. All of the tissue samples were homogenized by sonication and incubated on ice for 2 hours. After that, the homogenized tissue samples were centrifuged at 18,000xG for 30 minutes. Supernatant of the samples were aspirated and placed in fresh tube on ice. If necessary, the supernatant was centrifuged again (18,000xG for 30 minutes) to obtain clean tissue lysate and the pellet discarded. The tissue samples were aliquoted and kept in -80°C for future use.

The gel electrophoresis for the tissue samples were performed by preparing 25ml of NuPage® Tris-Acetate SDS running buffers (Invitrogen Ltd) in distilled water (500ml). Then, antioxidants (500µl) (Invitrogen Ltd) were added to the running buffer solution (200ml). The XCell Surelock™ Mini-Cell electrophoresis system (Invitrogen Ltd) was washed with water and distilled water, and properly dried. The NuPAGE® Novex 3-8% Tris-Acetate 1.0mm gel

(Invitrogen Ltd) was washed with distilled water and the comb was removed. The gel was placed in the electrophoresis tank and filled with the running buffer-antioxidant solution. After that, tissue samples were loaded into the wells of the gel together with the HiMark™ Pre-Stained protein standard (Invitrogen Ltd). The remainder of the electrophoresis system tank was filled with the running buffer prepared earlier. The lid for the electrophoresis apparatus was secured and connected to a power supply before running the tissue samples for 1 hour at 150V and 55mA at room temperature.

For the next step, the transfer buffer was prepared accordingly. The transfer buffer: 30mM Tris Base; 0.2M Glycine; 0.2% (w/v) SDS and 10% (v/v) Methanol in distilled water (500ml). Once the electrophoresis has stopped, the gel was removed from the apparatus and prepared for the protein blotting. Using the wet transfer technique, the sponges, the filter papers and the nitrocellulose membranes of 0.45µm pore size (Invitrogen Ltd) were soaked in the transfer buffer. In the transfer cassette, the nitrocellulose membranes were placed on top of the gel and sandwiched between the sponge and filter papers. Any trapped air bubbles were removed and the transfer cassette was put in the apparatus. The transfer buffer was added to the gel-membrane sandwich and into the apparatus tank. The protein blotting process was set for 1 hour 15 minutes at 30V and 220mA at room temperature. After that, the protein blot was prepared for calpain-1 probing steps.

Prior to the next step, the milk for blocking in the probing steps was made. The 10% (w/v) blocking milk contains: Tris-Buffered Saline (TBS) (100ml); Tween 20 (300µl) and dried milk powder (10g). After the protein blotting process, the nitrocellulose membranes were immersed in 10% milk for blocking process and put on the mixer roller for 1 hour. Then,

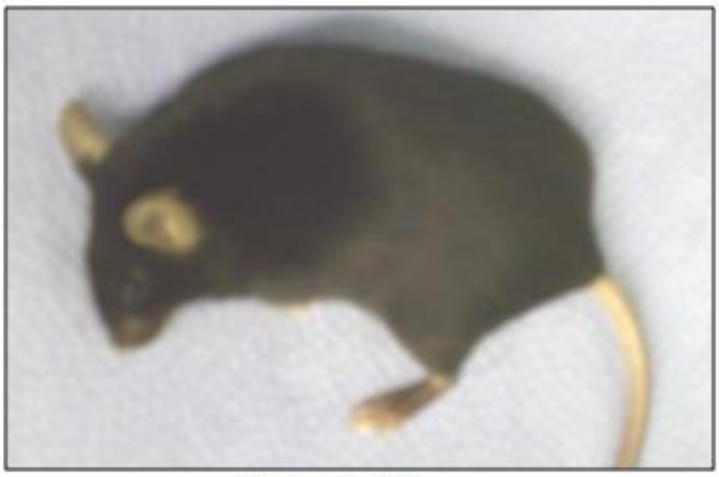
fresh 10% milk was added to the membranes and put in the fridge overnight. The next day, fresh 10% milk for blocking was added to the membranes and kept on the roller for 1 hour. Next, the membranes were washed three times (10 minutes each time) with 3% blocking milk. The membranes were probed with mouse anti-calpain-1 and anti-calpain-2 primary antibody (Santa Cruz Biotechnology) in 3% (w/v) milk for blocking (5µl calpain-1 antibody in 5ml of 3% blocking milk) and put on the mixer roller for 1 hour. This primary antibody is known to bind to the large subunit of calpain-1 and could also react to its smaller subunit (calpain-4). After that, the membranes were washed three times (10 minutes each time) with 3% milk for blocking purpose. An anti-mouse IgG-HRP secondary antibody (Santa Cruz Biotechnology) was added to the membranes (5µl secondary antibody in 5ml of 3% blocking milk) and kept on a roller for 1 hour. Subsequently, the membranes were washed twice with Tween-TBS composition and followed with TBS (for 10 minutes each time). The presence of calpain-1, calpain-2 and calpain-4 was detected using the SuperSignal West Pico Chemiluminescent substrate (Fisher Scientific) by immersing the solution with the membranes and then put on the mixer roller for 5 minutes. The membranes were exposed using UVIprochemi imaging system (UVItec Ltd), and images of the enzymes expressions on the membranes were captured.

3.4. Results

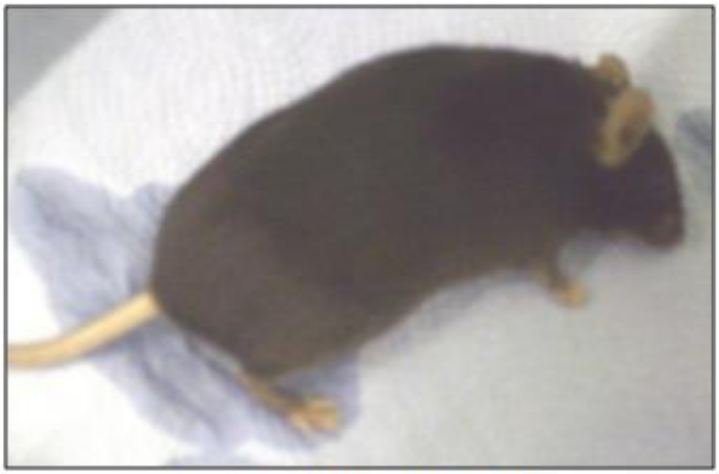
3.4.1. Phenotype Similarity and Physical Condition of the Mouse

The pups generated from either the normal wild-type, homozygous or heterozygous breeders appeared to be in good health. However, after several weeks in the sterile isolator barrier, one of the heterozygous female breeders was found dead without signs of illness. In addition, a 7-week old male homozygous calpain-1 KO mouse of the first line of generation happened to be suffering from a condition called hydrocephalus and had to be sacrificed. In order to determine the viability in a normal environment, a pair of heterozygous breeders was placed in the filter top (semi-clean) cages and showed that they could survive in such conditions and at the same time produce healthy pups. The homozygous and normal wild-type breeders put in the filter top cages were also shown to be healthy and able to produce healthy pups.

Close assessment of the mice showed that heterozygous, homozygous and normal wild-type mice had no phenotypical differences (Figure 3.4.1.1). Typically, mice at the age of 9-week old had similar sizes and weights (between 28 to 30 grams). Both homozygous and heterozygous mice had normal body shapes and could move actively as normal wild-type mice. Given the fact that the mice were generated from C57BL/6 strain which is generally black, the subsequent generations of mice appeared to have the same black coloured fur all over their body. The appearance of well groomed fur is also a sign that the mice were in healthy states. In addition, the mice did not have strict diet requirements and survived on usual pellet food and water. Thus, phenotypic variation was not present between the mice and the only approach to set them apart was by genotyping.



Normal wild-type



Heterozygous calpain-1 KO

Homozygous calpain-1 KO

Figure 3.4.1.1: Physical appearance of the normal wild-type, heterozygous and homozygous mice. This figure shows the physical appearance of normal wild-type, heterozygous and homozygous mouse generated from their respective breeding pairs.

3.4.2. Genotypic Variation of the Mouse Colony

The PCR procedure to genotype DNA samples from the mice was employed through the gene-specific or endogenous primers which lies outside of, and adjacent to the targeting vector arm. The bands from the gels electrophoresis are illustrated by the two different reactions; the endogenous primer GS[E] has only the endogenous reaction, and GS[E,T] has both of the endogenous and targeted reactions. The targeted alleles are visible at 314 base pairs which was due to the reactions between GS[E,T] primer and the targeting vector (NEO[T]) primer. The endogenous alleles are detected with the reaction between GS[E] and GS[E,T] primers at 400 base pairs. The results are demonstrated by different base pairs observed on the multiplex lane (with GS[E], GS[E,T] and NEO[T] primers) and on the endogenous lane (with GS[E] and GS[E,T] primers). The DNA samples with two bands at 400 base pairs on the multiplex and endogenous lane represent the normal wild-type mouse. The bands visible at the region of 300 and 400 base pairs on multiplex lane together with one band at 400 base pairs on the endogenous lane represent the heterozygous mouse (Figure 3.4.2.1a). The homozygous KO mice were identified by the presence of one band at 300 base pairs region on the multiplex lane.

The image in Figure 3.4.2.1b shows genotyping results from the very first generation of pups produced by the heterozygous breeding pairs. Three male (number 1, 2 and 3) and two female mice (number 8 and 9) are homozygous calpain-1 KO; one male (number 7) and female (number 10) heterozygous calpain-1 KO; and three male of normal wild-type mouse (number 4, 5 and 6). The genotype results from the pups of the homozygous breeding pairs confirmed the first generation of healthy homozygous calpain-1 KO mice (Figure 3.4.2.1c).

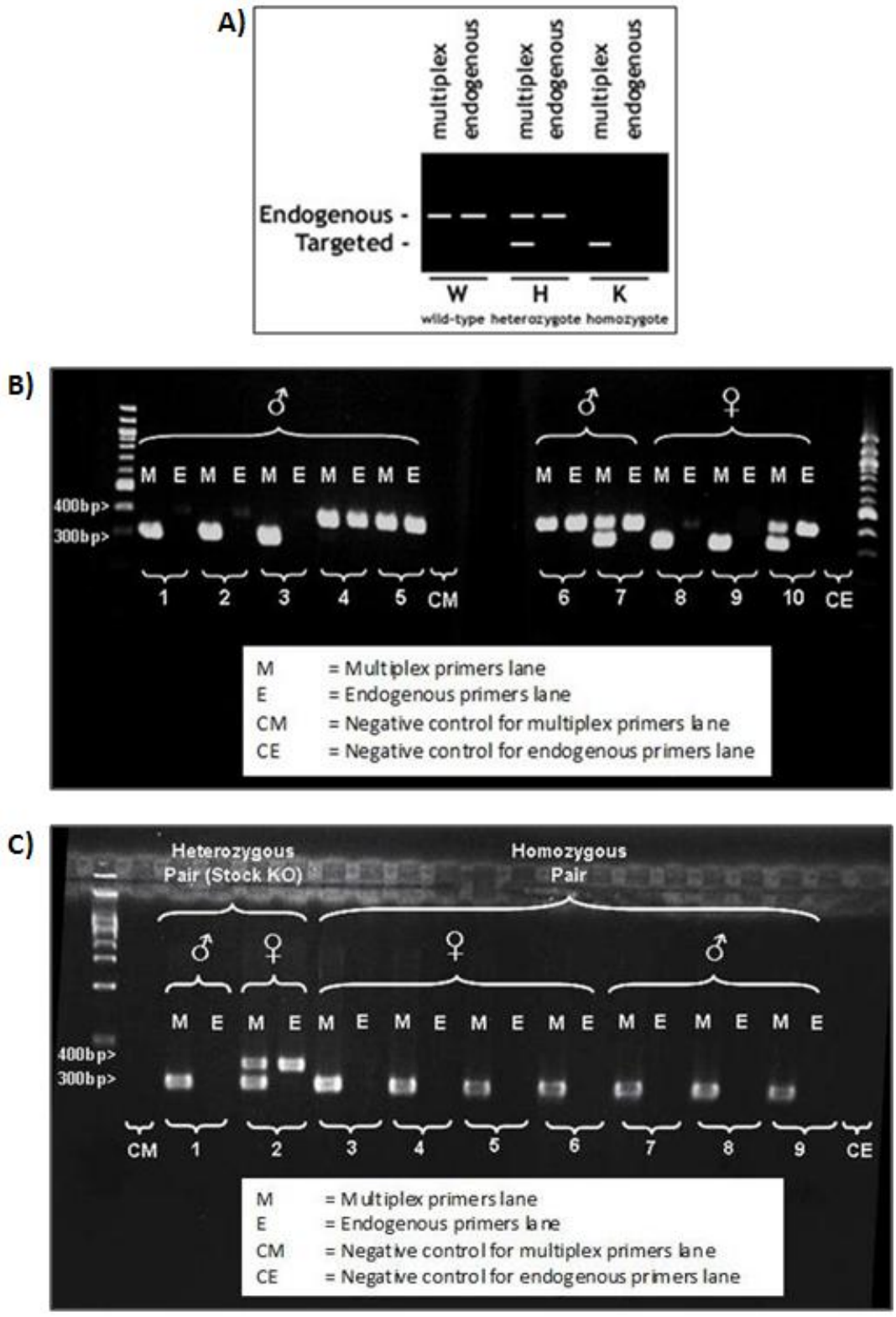


Figure 3.4.2.1: Results interpretation for mouse genotyping. Image (A) describes the results analysis for genotyping. Image in (B) is the genotyping result of the first batch of pups generated through heterozygous breeders; homozygous mice: 1, 2, 3, 8 and 9; heterozygous mice: 7 and 10; normal wild-type mice: 4, 5 and 6. Image (C) is the genotyping result from the first generation of homozygous calpain-1 KO pups produced by homozygous breeders; homozygous mice: number 3 to 9.

3.4.3. Expression of Calpain-1 in the Tissue Samples

The presence of calpain-1 in other tissues was examined using the Western blotting protocol. This has showed high expression of the enzyme in the RBC, bone marrow and liver cells taken from normal wild-type mice. By using the primary antibody of calpain-1 which reacts to the enzyme's large catalytic and small regulatory subunit, the molecular weight of these subunits in normal wild-type samples were detected at 80kDa and 30kDa respectively (Figure 5.4.2.1). Adding the secondary antibody showed the presence of IgG in these same tissue samples. On the other hand, the homozygous calpain-1 KO samples also showed bands of 80kDa and 30kDa molecular weights, except with lower expression as compared to the normal wild-type samples (Figure 5.4.2.1).

This antibody recognised both calpain-1 and calpain-4 (small regulatory subunit) and thus this finding probably resulted from a cross reactivity with calpain-2 enzyme. Therefore, the same Western blotting protocol using calpain-2 as the primary antibody was performed on the samples. The blotting results presented similar effects, the expressions of calpain-2 appeared to be higher at the 80kDa and 30kDa bands in the normal wild-type mice samples than in the homozygous calpain-1 KO (Figure 5.4.2.2). Surprisingly these results showed that the expressions of calpain-4 and calpain-2 enzyme in the homozygous calpain-1 KO samples were low. This signifies that the calpain-1 gene deletion might have affected the production of calpain-4 and calpain-2 enzymes.

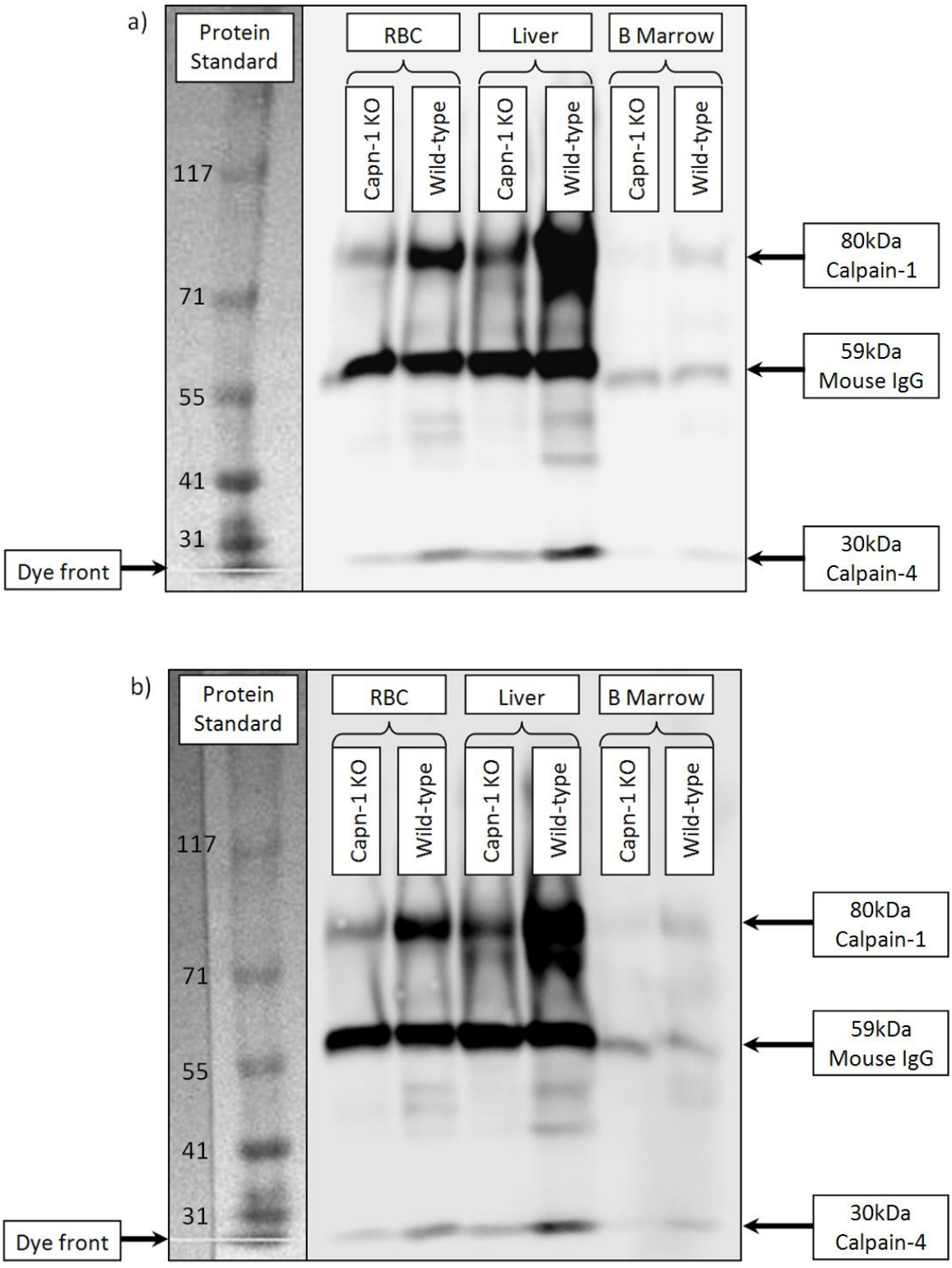


Figure 3.4.3.1: The western blotting results of calpain-1 and calpain-4 expression. This figure shows western blotting results of calpain-1 and calpain-4 expressions in the RBC, liver and bone marrow samples. Expression of calpain-1 in normal wild-type tissue samples appeared to be higher than the homozygous calpain-1 KO samples. The results also indicated that calpain-4 expressions in the homozygous calpain-1 KO tissue samples are lower and could have been affected by the calpain-1 knock-out.

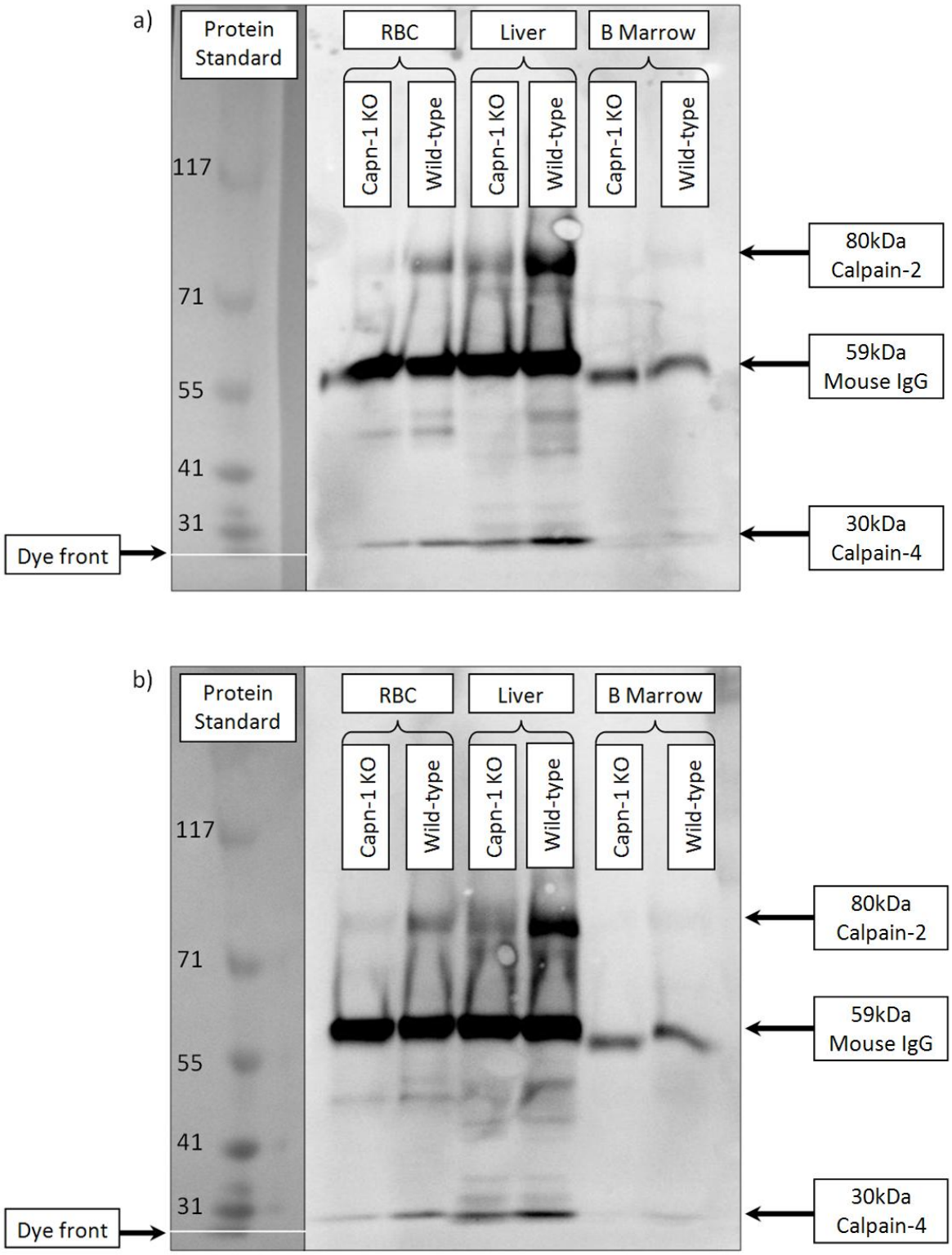


Figure 3.4.3.2: The western blotting results of calpain-2 and calpain-4 expression. The images above show western blotting results of calpain-2 and calpain-4 expression in the RBC, liver and bone marrow samples. The calpain-2 expression in normal wild-type tissue samples appeared to be high, but exhibit diminishing effects in homozygous calpain-1 KO samples. The results also suggested that the production of calpain-2 in homozygous calpain-1 KO mice could have been affected due to the calpain-1 knock-out.

3.5. Discussion

The work presented in this chapter proved that generating homozygous calpain-1 KO mice through the selective breeding plan has been a success. In general, the mice appeared to be in good health although there have been an unfortunate case of the pup having health problems early on. The hydrocephalus condition discovered in one of the homozygous mice was a common condition that could develop in the normal C57BL/6 wild-type mouse strain (Green et al. 2007). The mice colony demonstrated good adaptability to their environment and could survive on regular pellet food and water. Characteristically there was no evidence of any variation between the features on the normal wild-type C57BL/6 mice with either heterozygous or homozygous calpain-1 KO mice. Thus, interpreting the genotyping results and implementing a good ear coding categorization were the only alternative method available to differentiate the mice in order to attain the correct neutrophil samples.

It is also shown that calpain-1 was significantly reduced and is probably absent in the cells from calpain-1 gene deleted animals. However, some antibody binding was observed and this could be due to the cross-reactivity of the primary antibody towards calpain-2. One surprising finding was that the expression of calpain-4 was also reduced in the homozygous calpain-1 KO mice. This suggested that the calpain-1 knock-out may also have an effect on the production of calpain-4. The heterodimeric structure of calpain means that calpain-1 deficiency may have contributed to the diminishing presence of calpain-4. The production of unstable calpain-4 could also have a knock-down effect that decreases calpain-2 production. In general, the generation of homozygous calpain-1 KO mice could help to understand how calpain-1 regulates neutrophil behaviour and functions.

Chapter 4

The Role of Calpain-1 in Neutrophil Trans-endothelial Migration

4.1. Introduction

The generation of calpain-1 KO mice presented the opportunity to study the role of calpain-1 in regulating neutrophil function. Comparing the cellular reaction between normal wild-type and calpain-1 deficient neutrophils provide the possibility of detecting any defects due to the absence of calpain-1. The subject of the work in this chapter explores the effects of calpain-1 deficiency in neutrophils trans-endothelial migration ability *in vivo* and *in vitro*.

4.1.1. Neutrophil Trans-endothelial Migration

Neutrophil recruitment from the blood to the extravascular space is one of the key actions for antibacterial function. It is well documented over the years that this movement involving chemoattractants requires the cells to spread and cross through the blood vessel endothelial cell lining in a process known as trans-endothelial migration. The success of this process depends on the molecular interaction between integrin molecules on the neutrophil surface and the intercellular adhesion molecule-1 (ICAM-1). It has been demonstrated that in inflammation both CD11b and lymphocyte function-associated antigen-1 (LFA-1) together with ICAM-1 played prominent roles in the trans-endothelial migration process (Shaw et al., 2004; Yang et al., 2005). Neutrophil CD11b/CD18 integrin has been shown to undergo rapid reorganization during transmigration and is distributed at the point of contacts with the endothelial cells junctions, the latter being enriched with ICAM-1. It is proposed that the molecular affinity of LFA-1 towards the endothelial cell ICAM-1 ligand indicates the interaction that happens during the transmigration process.

In addition, pre-treatment of the endothelial cells with TNF-α has profound effects on neutrophil adherence and transmigration. Some of the previous studies suggested that TNF-α caused neutrophils to firmly adhere onto the coated surface making them incapable of detaching (Salyer et al., 1990), as well as inhibiting cell polarization and chemotaxis (Lokuta and Huttenlocher, 2005). Interestingly, when neutrophils in suspension were added on to the endothelial cells pre-treated with TNF-α, it showed that their migration across the cellular barrier were greatly increased (Bombini et al., 2004; Furie and McHugh, 1989; Smart and Casale, 1994). Pre-treatment of endothelial cells with TNF-α increased the expression of ICAM-1, and this has been identified as one of the essential molecules that promotes neutrophil transmigration across the barrier (Yang et al. 2005). Therefore, the capability of neutrophils from normal wild-type and homozygous calpain-1 KO mice to migrate across the endothelial cell barrier with TNF-α treatment *in vitro*, and the intraperitoneal recruitment by gastric lavage *in vivo*, were investigated.

4.2. Aims of the Chapter

The aims of the work described in this chapter were therefore to:

1. Quantitatively determine neutrophil *in vivo* recruitments in the peritoneal cavity of the normal wild-type and homozygous calpain-1 KO mice following zymosan injection.
2. Analyse *in vitro* differences between transmigrated neutrophils of normal wild-type and homozygous calpain-1 KO mice following the fMLP gradient, and the TNF-α effects.
3. Detect the presence of integrin on the surface of normal wild-type and homozygous calpain-1 KO neutrophils.

4.3. Methods

This methodology section describes the protocol and technique used to perform the experimental works for this chapter. The methods include; counting the numbers of normal wild-type and homozygous calpain-1 KO neutrophils that have transmigrated across the barrier created by the endothelial cells *in vitro*, and quantifying the number of neutrophils recruited into the peritoneal cavity of normal wild-type and homozygous calpain-1 KO mice *in vivo* by injecting zymosan particles.

4.3.1. *In vivo* Neutrophil Recruitment to the Peritoneal Cavity

Female mice between 9-10 weeks old were randomly selected for this work. All of the proper care and management of the animals were exercised during this experimental procedure. Prior to the experiment, the zymosan particles were prepared by diluting in PBS ($2x10^8$/ml). The *in vivo* recruitment of the normal wild-type and homozygous calpain-1 KO neutrophils was then accomplished by injecting zymosan particles (100µl) into the peritoneal cavity of the mice. The introduction of the foreign substance leads to the recruit of neutrophils at the site of injection. Based on data of from initial trials, neutrophil recruitment to the intraperitoneal location reached a maximum after 6 hours. Therefore, in order to study the transmigration rate, the mice were left for 2 hours after the intraperitoneal injection before they were sacrificed, gastric lavage performed and neutrophils were collected. For the gastric lavage, a syringe was used to inject PBS solution (3ml). Neutrophils in PBS solutions were then collected into syringes and put on ice before the cell quantification step (Figure 4.3.1.1).

The quantification of neutrophil recruitment was performed using a fluorescence activated cell sorting (FACS) machine (Beckman Coulter Ltd). The FACS solution was prepared by diluting the cell samples (100µl) with the inflammatory marker antibodies (50µl), and the Fc receptor blocking reagent (50µl) in order to reduce background stains. The Gr-1-V450 and Ly-6B-APC antibodies (BD Biosciences) were used as the granulocyte and neutrophil markers respectively, and together with fluorescence beads (BD Biosciences) were used for quantification. The cells were quantified via the violet-2 channel of the FACS machine and the results were recorded. The scattered plot graph of neutrophils that had been recruited into the peritoneal cavity were identified and analysed. The percentages and the number of transmigrated neutrophils were calculated accordingly.

The data are reported as mean +/- S.E.M. The means from different experiments were compared by one-way analysis of variance. When significant differences were identified, the comparisons were subsequently made using the Student's *t*-test for unpaired values. Statistical significance was set at $p<0.05$.

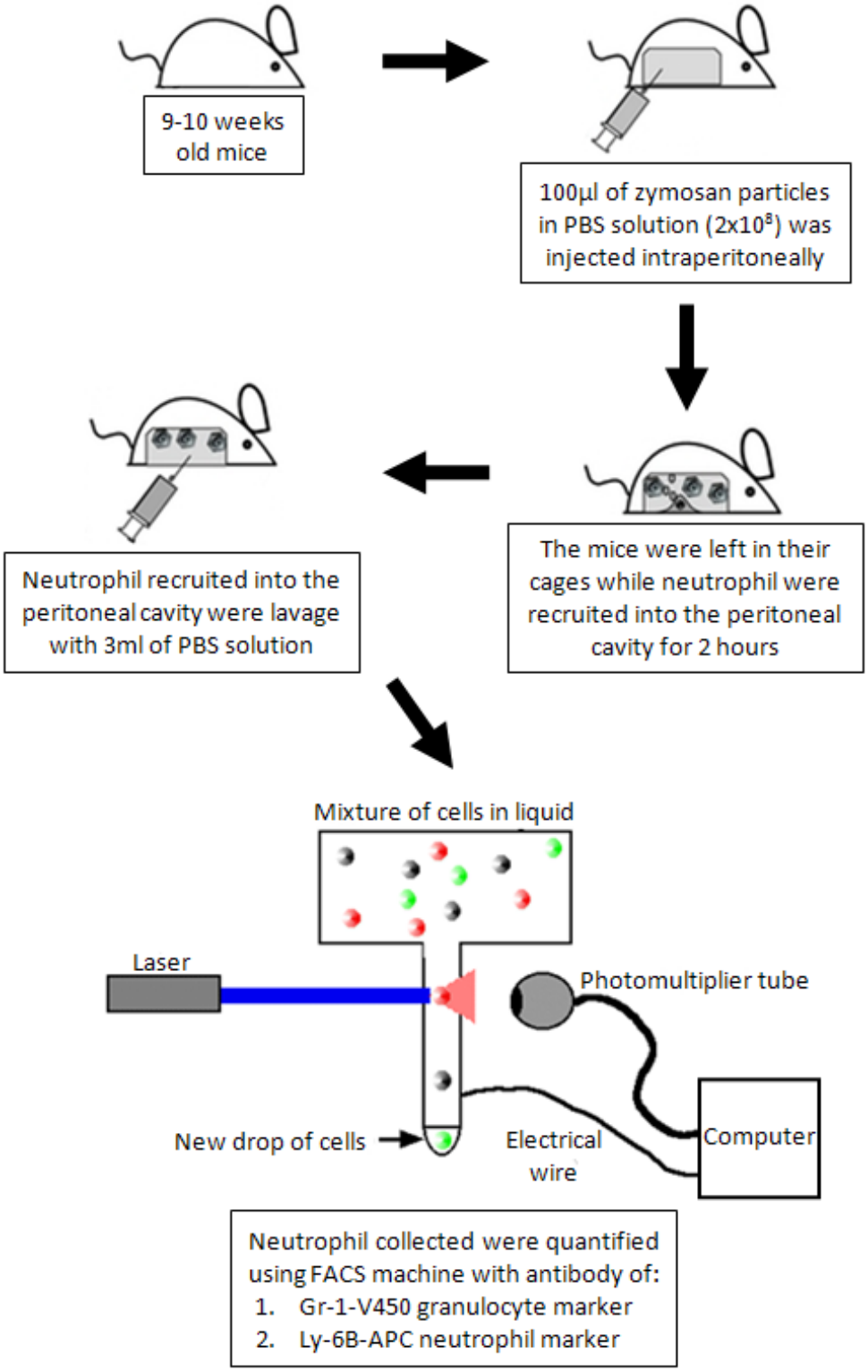


Figure 4.3.1.1: Experimental procedure for in vivo recruitment of neutrophils. The flow chart explains the experimental procedure for *in vivo* recruitment of neutrophils in the mice. Neutrophils were recruited through zymosan intraperitoneal injection and their numbers were quantified using the FACS machine.

4.3.2. Culturing and Treating Endothelial Cells for *In vitro* Transmigration Assay

The human umbilical vein endothelial cells grown in the culture flasks for about one week were seeded in the cell culture inserts with 3µm pore size and grown with Roswell Park Memorial Institute (RPMI) medium. The inserts were placed in 24 well plates to create the monolayer barrier. The 24 well plates with the endothelial cells were incubated at 37°C with 5% CO_2 for about 3 to 4 days in order to achieve desirable confluency. After the incubation period, the medium in the inserts and wells were replaced with fresh RPMI medium. As for the wells designated for TNF-α treatment, fresh medium with TNF-α were added into the inserts. The plates were then incubated (37°C with 5% CO_2) for another 30 minutes. Prior to the assays, the endothelial cells treated with TNF-α were washed twice by aspirating the whole medium before replacing it fresh medium (100µl) into the inserts and into the wells (500µl). This final step was repeated on the inserts and wells of the non-treated endothelial cells. The plates were then prepared for the transmigration assay.

4.3.3. *In vitro* Transmigration Assay

Circulating blood neutrophils from the normal wild-type and homozygous calpain-1 KO mice were isolated and suspended in RPMI medium. Using the UpCell™ Surface 24 well plates with the treated and non-treated human endothelial cells inserts prepared from the previous step, neutrophils suspended in HBK (100µl) were added into each of the inserts before being incubated for 30 minutes (Figure 4.3.3.1). This incubation step is to promote molecular interactions between neutrophils and ICAM-1 on the surface of the endothelial cells. Next, medium in the wells of the plates was aspirated and replaced with 0.5µM fMLP

solution in RPMI medium (500µl). The fMLP solution acts as chemoattractant which created a concentration gradient emanating from the bottom of the 24 well plates but limited by the endothelial cells layer in the inserts. The UpCell™ Surface plates and the insert set-ups were incubated for up to 6 hours at 37°C in 5% CO_2. At the end of the incubation period, the inserts were removed and the UpCell™ Surface plates were left at room temperature for several minutes. This step caused changes in temperature to the polymer surface in the wells of the plates and allows transmigrated neutrophils that have attached to the bottom of the wells to be released. Neutrophils that have transmigrated successfully through the endothelial cells were collected and counted using a haemocytometer. The cells were fixed with 4% (v/v) formaldehyde for 30 minutes and stained using Hemacolor® staining kit (Merck Chemicals, UK). The data are reported as mean +/- S.E.M. The means from different experiments were compared by one-way analysis of variance. The comparisons were made using Student's *t*-test for unpaired values. Statistical significance was set at $p<0.05$.

4.3.4. Integrin Immunofluorescent Staining

This integrin immunofluorescent staining was carried out using Anti-CD11b antibody conjugated to FITC (Abcam, UK). The antibody was pre-incubated with suspended neutrophils in HBK medium in 1:1000 µl ratios for 30 minutes. After that, neutrophils in the suspension (100µl) were put on the glass coverslip to adhere. Any unbound cells were then removed by washing them twice with HBK. Neutrophils labelled with immunofluorescence integrin antibody in the normal wild-type and homozygous calpain-1 KO sample were identified using the inverted confocal laser scanning microscope, and the images were taken for comparison.

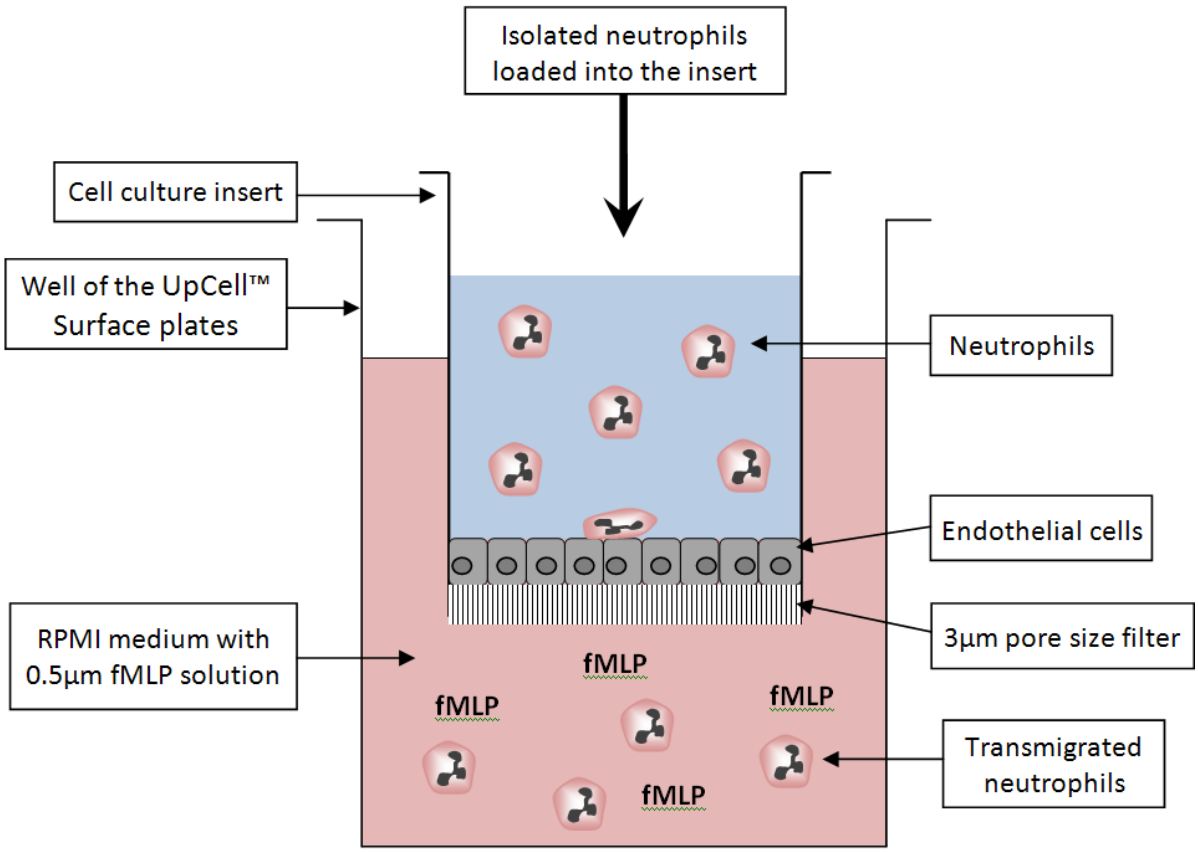


Figure 4.3.3.1: *In vitro* experiment for the neutrophil trans-endothelial migration assay. The endothelial cells were grown in the inserts and placed in 24 well UpCell™ Surface plates filled with 0.5µM fMLP solution in RPMI medium. Neutrophils that have successfully transmigrated will accumulate in the wells at the bottom before they were collected and counted.

4.4. Results

4.4.1. Neutrophil Recruitment In vivo

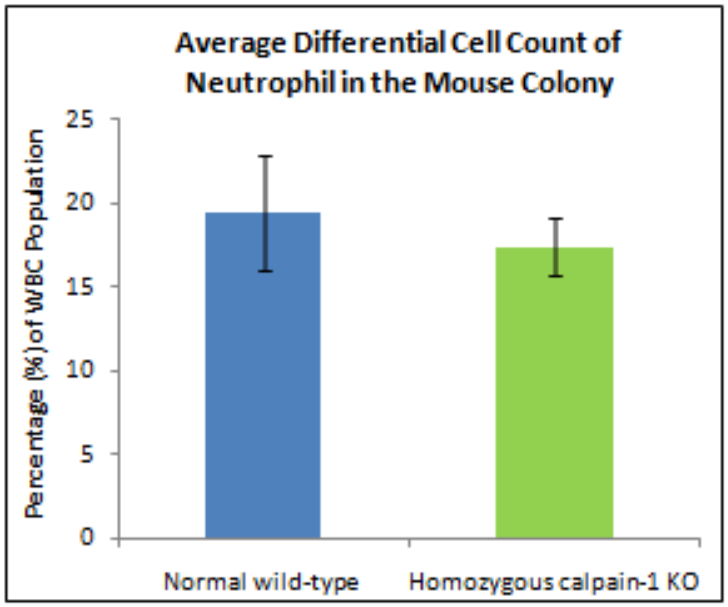
In order to discover whether the absence of calpain-1 had a significant effect on the recruitment of neutrophils to an inflammatory site, an *in vivo* approach was adopted. The outcome from this *in vivo* experiment in the normal wild-type and homozygous calpain-1 KO mice would give an insight into the ability of neutrophils to migrate to their target site in a normal and inflammed condition. Data from neutrophil differential cell counts and absolute cell counts in the mice showed that the numbers of circulating blood neutrophils were similar in both the normal wild-type and homozygous calpain-1 KO animals (Figure 4.4.1.1). The average percentage of neutrophils in the peritoneal fluid quantified by using the FACS machine was 52.3% of the WBC population for normal wild-type mice, as compared to only 35.8% of the WBC population in the homozygous calpain-1 KO animals (Figure 4.4.1.2 and 4.4.1.3). The outcome shows that when challenged to migrate *in vivo*, homozygous calpain-1 KO neutrophils were only capable of undergoing transmigration at reduced rate after 2 hours of intraperitoneal injection. Average neutrophil counts were calculated based on the number of fluorescent beads (10 900) from the stock (1ml) and the numerical factor of the antibody employed (60). The results showed $3.0x10^6$ cells have been recruited into the peritoneal cavities of normal wild-type mice, compared to only $1.8x10^6$ cells in homozygous calpain-1 KO mice (Figure 4.4.1.3). The cell counts were significantly different between the normal wild-type and homozygous calpain-1 KO mice.

This *in vivo* effect suggests that the ability of homozygous calpain-1 KO neutrophils to transmigrate when induced through complement activation by intraperitoneal zymosan

has been disrupted. It means that without calpain-1, neutrophil transmigration ability within the homozygous calpain-1 KO mice has been disturbed. However, the calpain-1 knock-out procedure may have an accompanying effect that causes a defect to the endothelial cells as well. Thus, the ability of normal wild-type and homozygous calpain-1 KO neutrophils to transmigrate was further investigated on normal endothelial cell monolayers using an *in vitro* trans-endothelial migration model.

As part of an ongoing study on inflammatory disorders, the mice were also used in a preliminary antigen-induced arthritis experiment. Arthritis was induced in the normal wild-type and homozygous calpain-1 KO mice by intra-articular injection of mBSA (methylated bovine serum albumin) in PBS (0.1 µg/joint) into the right knee joint. A day after induction, the knee joints of both normal wild-type and homozygous calpain-1 KO mice appeared swollen, and diameter of the swelling was measured. Preliminary results showed that the difference in average swelling diameter before and after the induction was not significantly different between the normal wild-type and homozygous calpain-1 KO mice measurement of 2.57mm (+/- 0.32 S.E.M) and 2.45mm (+/- 0.41 S.E.M) respectively. It is anticipated that the arthritic condition would reduce the number of circulating blood neutrophils due to the cells being recruited to the knee joint. Hence, the cell count of circulating blood neutrophils was carried out. Average cell count of normal wild-type neutrophils was recorded at 65.22 cells/µl (+/- 7.82 S.E.M) and homozygous calpain-1 KO neutrophils with 63.23 cells/µl (+/- 7.45 S.E.M), which again showed no significant differences. Therefore, it is expected that the following histological work on the knee joints taken from these mice would provide clearer answer on neutrophils transmigration ability in this experimentation model.

A)



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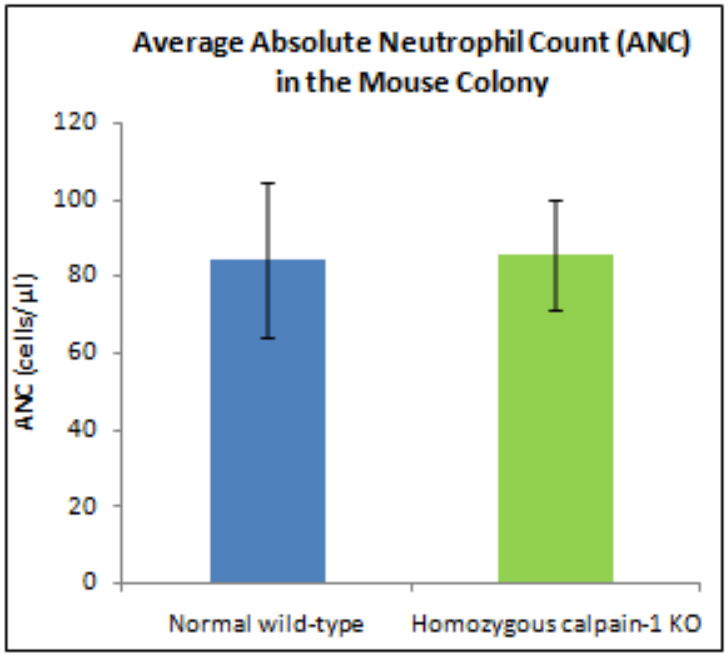
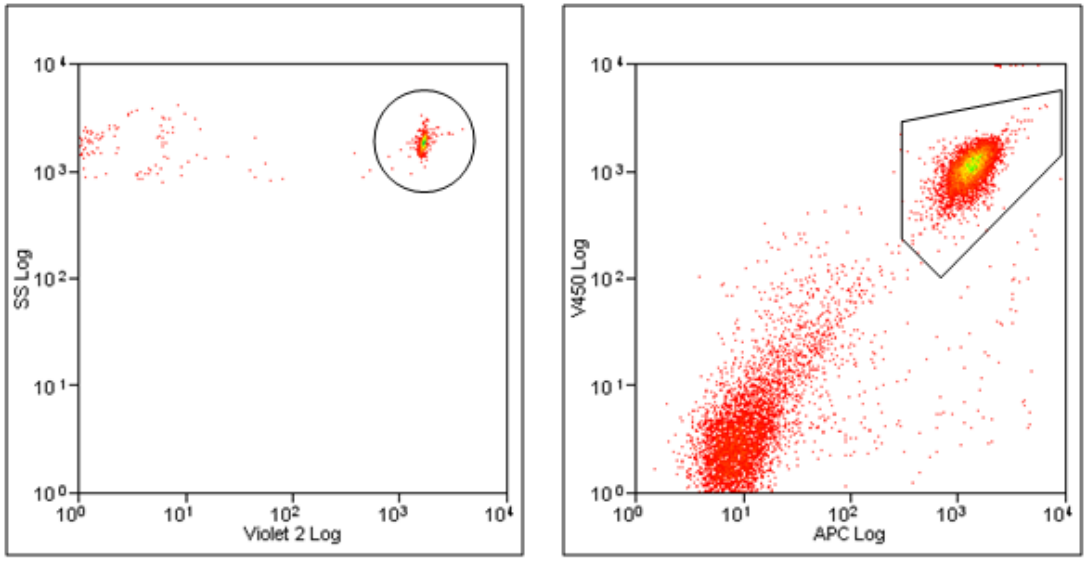


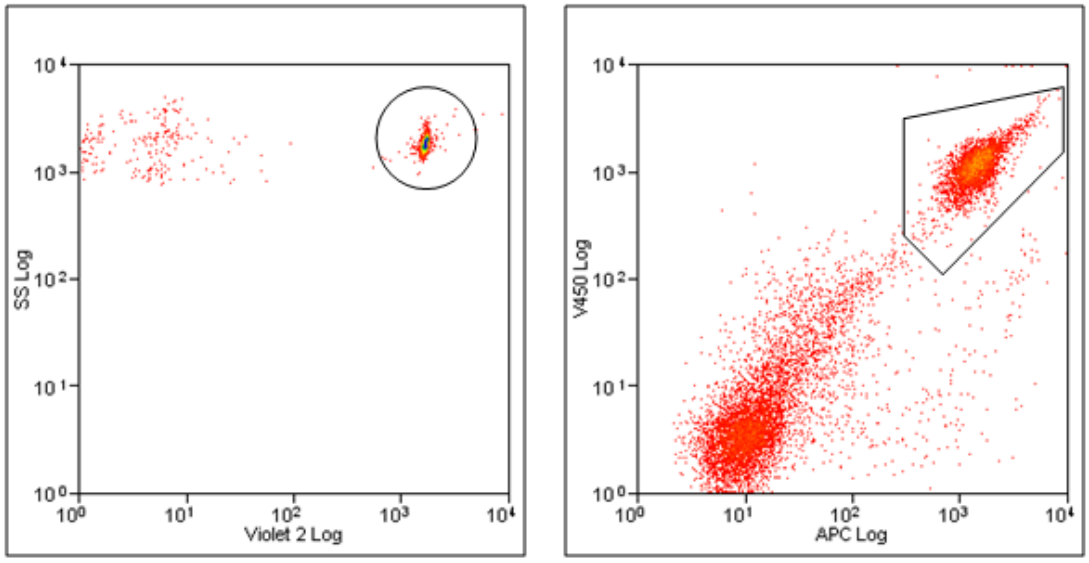
Figure 4.4.1.1: The average differential cell count and absolute neutrophil count. The graphs represent the average (A) differential cell count, and (B) absolute neutrophil count (ANC) of circulating blood neutrophils in the homozygous calpain-1 KO and normal wild-type mouse colony. The results showed a comparable number of neutrophils in the mice with no significant difference between them. This means that knocking-out calpain-1 did not affect the production of neutrophils in the mice.

Normal Wild-type Neutrophils Density Plot



Beads Counts Neutrophils Counts

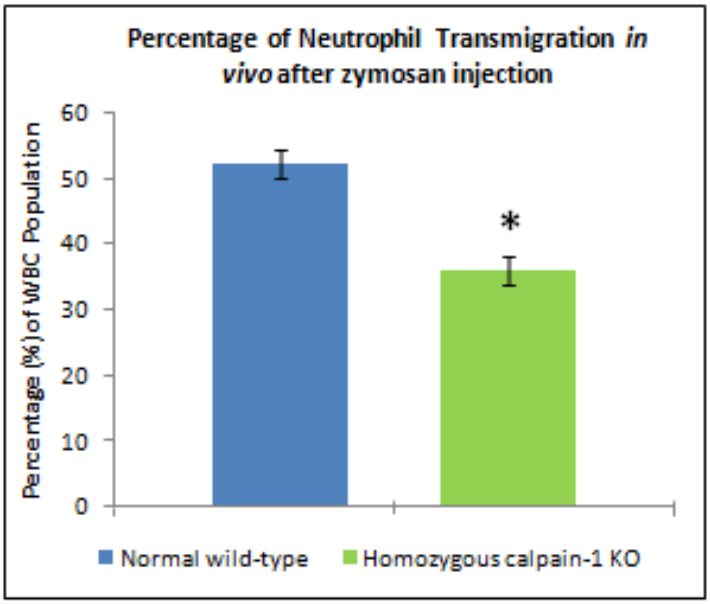
Homozygous Calpain-1 KO Neutrophils Density Plot



Beads Counts Neutrophils Counts

Figure 4.4.1.2: The density plots of neutrophil quantification in the FACS machine. The density plots above represent the neutrophil quantification through the gated channel in the FACS machine. The circled areas on the bead count plots represent the WBC population using Gr-1-V450 and Ly-6B-APC antibodies. The pentagon areas on the neutrophil count plots represent the neutrophil population (Gr-1-V450 antibody) and the other WBC population (outside the pentagon areas). By defining the dot plot areas (as shown in the plotted image), the percentage of granulocytes and neutrophils collected from the normal wild-type and homozygous calpain-1 KO mice were calculated accordingly.

A)



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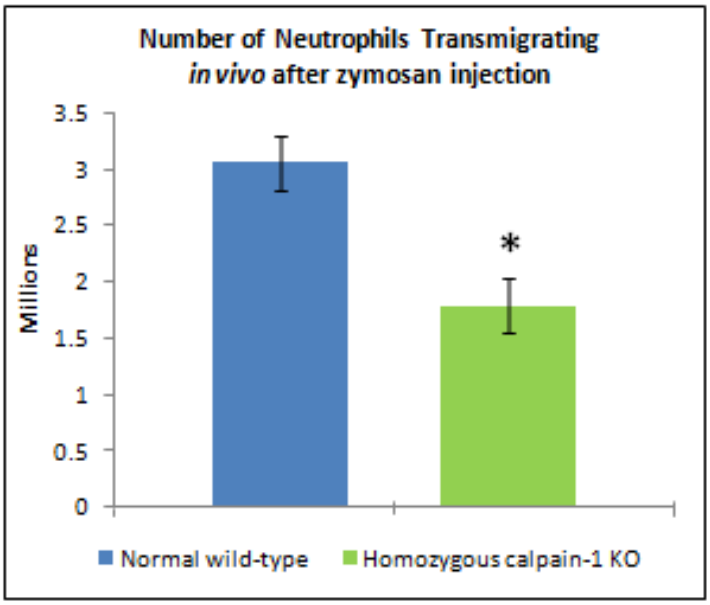


Figure 4.4.1.3: The percentage of neutrophil quantification using the FACS machine. The graph in (A) represents the percentage of neutrophils quantification using the FACS machine which showed a significant difference between normal wild-type and homozygous calpain-1 KO cells recruited into the peritoneal cavity of the mice. The graph in (B) shows the number of neutrophils counted through the specified gated channel (violet-2). The number of the cells is calculated based on the bead concentration (10 900 per µl), and numerical factor of the samples put through the machine (60µl). The number of neutrophils recruited into the peritoneal cavities of the normal wild-type and homozygous calpain-1 KO mice also showed significant difference between them. Results are expressed as mean +/- S.E.M. and are representative of two separate experiments. *p<0.05 compared to the normal wild-type mice (analysis of variance followed by Student's t-test).

4.4.2. Neutrophil *In vitro* Transmigration

Neutrophil's ability to transmigrate on TNF-α treated and non-treated endothelial cells showed an intriguing result. It was demonstrated that a large population of normal wild-type neutrophils successfully migrated across the TNF-α treated endothelial cell monolayer (Figure 4.4.2.1). This was in contrast to the homozygous calpain-1 KO neutrophils that were able to cross the cell barriers at a slower rate (Figure 4.4.2.2). In the absence of TNF-α, both calpain-1 deficient and normal wild-type neutrophils were able to cross the endothelial cells barrier, but at a greatly reduced rate as compared to the endothelial cells treated with TNF-α. This result showed that the defect in calpain-1 deficient neutrophils was restricted to the presence of TNF-α on the endothelial cells. The difference between TNF-α treatment and non-treatment was a useful indicator for the trans-endothelial migration rate. Since homozygous calpain-1 KO neutrophils did not show any significant differences in transmigration on either of the TNF-α treated or non-treated endothelial cells, it was concluded that the trans-endothelial migration was inhibited by calpain-1 deficiency.

In order to quantify transmigration competency, the cell populations from normal wild-type and homozygous calpain-1 KO samples were counted using the haemocytometer (0.1mm depth) and expressed as a percentage of the total starting cell numbers. This showed that there was a significant difference ($p<0.05$) in transmigration ability as normal wild-type neutrophils exhibited a higher percentage of transmigrated cells than the homozygous calpain-1 KO cells (Figure 4.4.2.3). Again, this striking contrast was only evident with neutrophils (normal wild-type and homozygous calpain-1 KO) transmigrating on TNF-α treated endothelial cells and confirmed the effects seen under the microscope.

Normal Wild-type Neutrophils

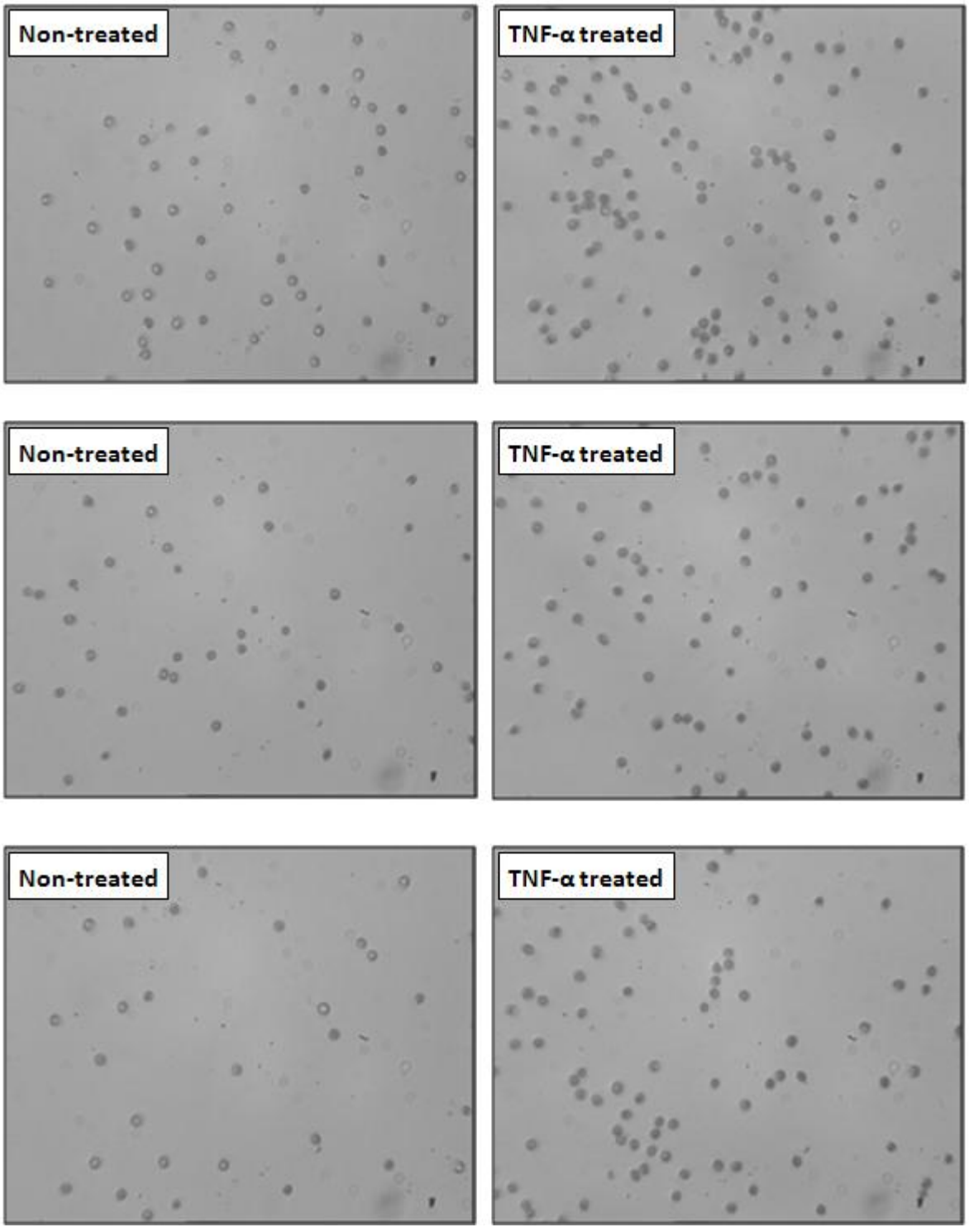


Figure 4.4.2.1: The number of transmigrated normal wild-type neutrophils after the 6 hour incubation period. The pictures show the number of transmigrated normal wild-type neutrophil after 6 hours incubation period. Visibly, transmigrated neutrophil have bigger population in the TNF-α treated endothelial cells condition. This was confirmed through cells counts of the transmigrated neutrophil with and without the TNF-α treatment.

Homozygous Calpain-1 KO Neutrophils

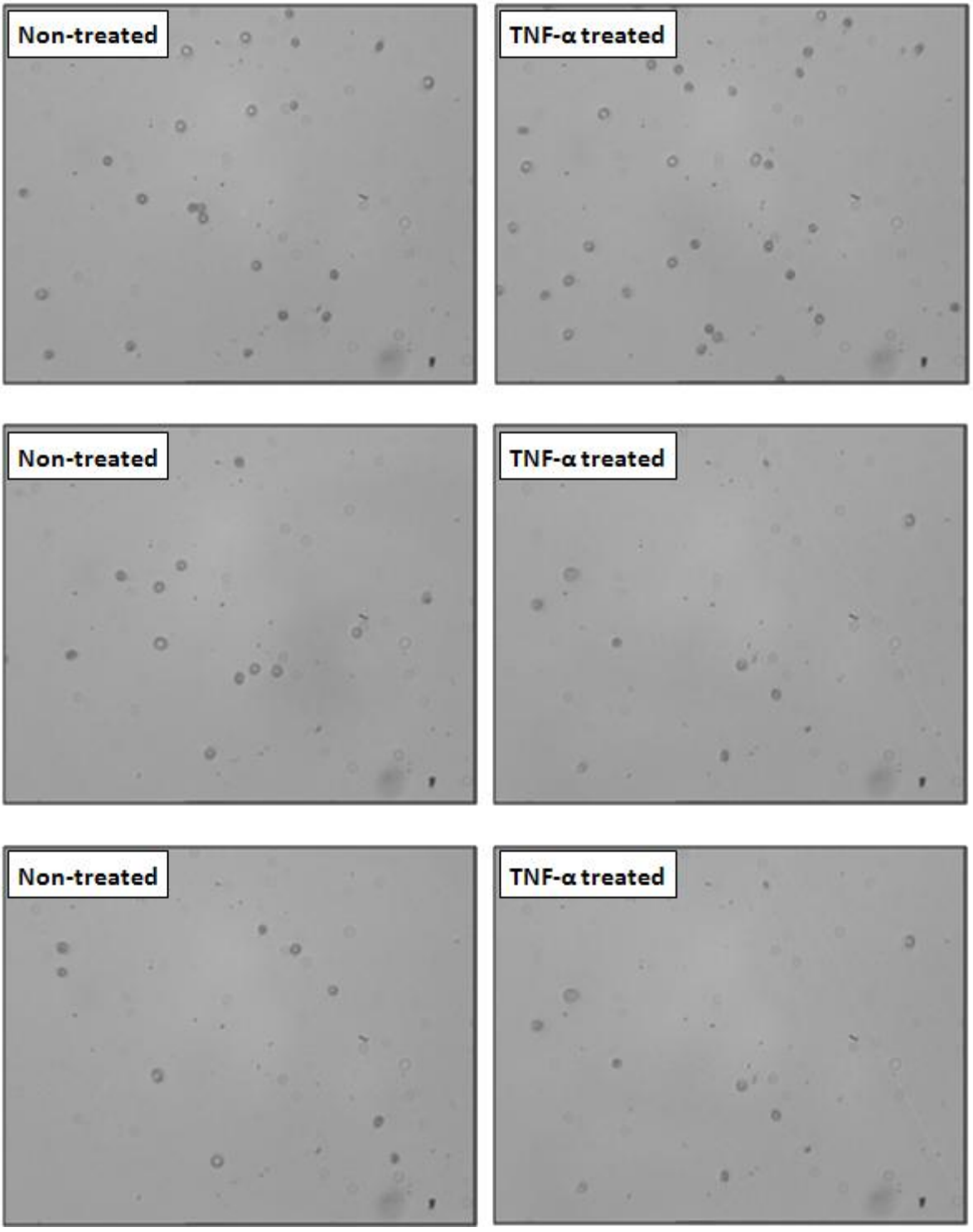


Figure 4.4.2.2: The transmigrated neutrophils of the homozygous calpain-1 KO mice after the 6 hour incubation. In general, this figure showed that the population of transmigrated neutrophil on either TNF-α treated or non-treated endothelial cell were similar. This was verified by the cells counts, which implied that TNF-α did not influence the transmigration process. However, the effects showed a striking difference in comparison to the transmigrated normal wild-type neutrophil in Figure 4.4.2.1.

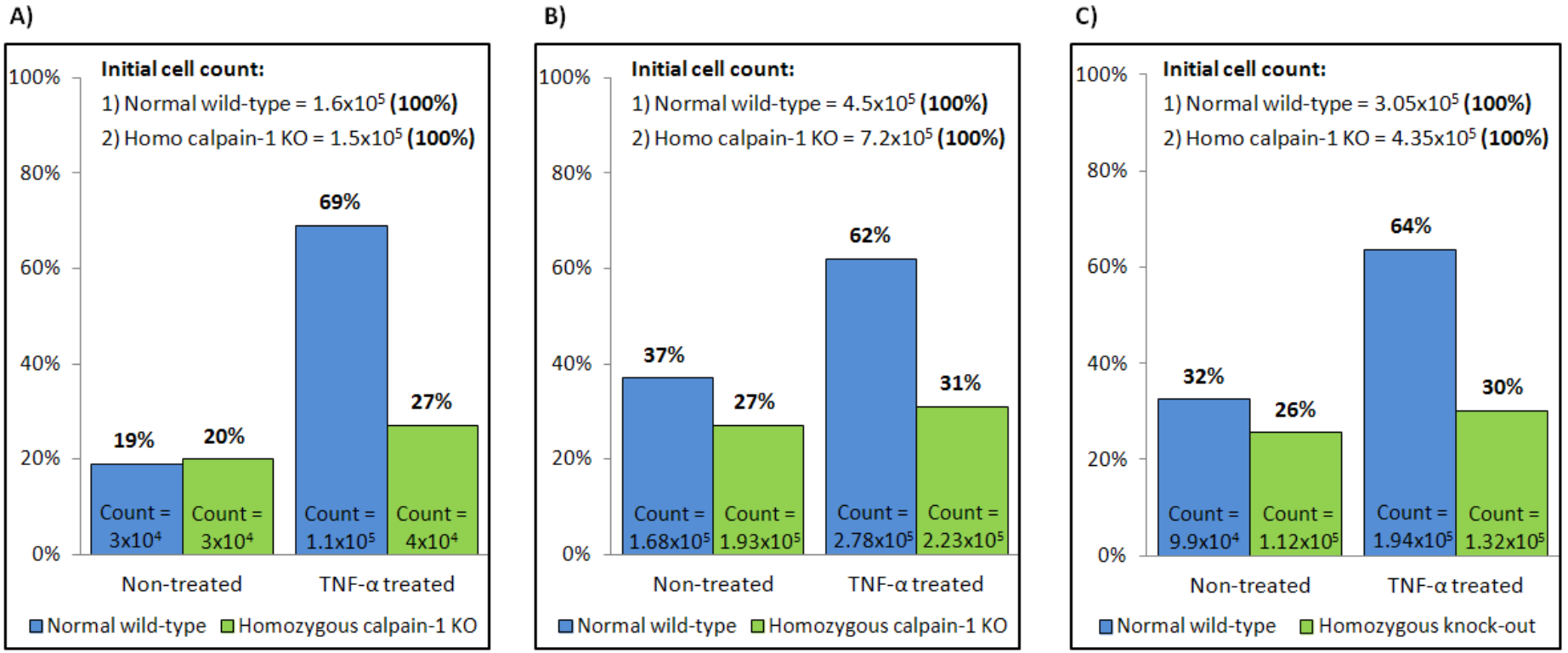
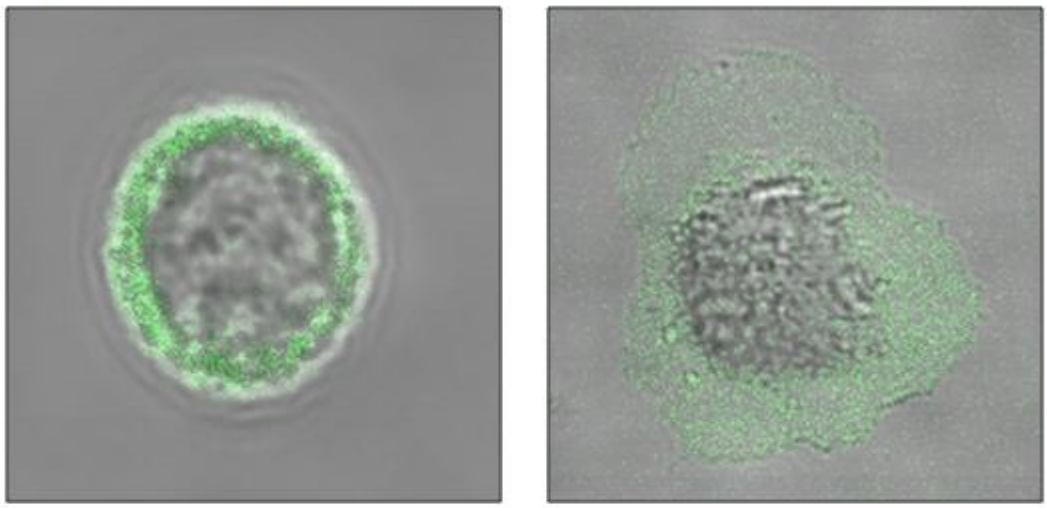


Figure 4.4.2.3: The result of trans-endothelial migration experiments. The graphs represent the raw data from three separate trans-endothelial migration experiments. Transmigrated neutrophils from normal wild-type and homozygous calpain-1 KO mice (in TNF-α treated and non-treated endothelial cells) were counted with a haemocytometer. The cell counts of transmigrated neutrophil populations were calculated against the initial cell counts at the beginning of every experiment and the calculations are represented in percentages. The results showed similar transmigration trends with higher numbers of normal wild-type cells in TNF-α treated conditions and a marked difference in comparison to the homozygous calpain-1 KO cell counts. In addition, the TNF-α effect on homozygous calpain-1 KO neutrophils only gives marginal cell count differences as compared to either normal wild-type or homozygous calpain-1 KO neutrophils without TNF-α. This result is grouped together and presented by their average counts as presented in Figure 4.4.2.5.

Further analysis on the number of transmigrated cells confirmed the effect of TNF-α treatment, and the defect in homozygous calpain-1 KO neutrophil trans-endothelial migration. The presence of integrins on the surface of homozygous calpain-1 KO neutrophils as compared to normal wild-type neutrophils, proved that the defect was not due to lack of CD11b/18 (Figure 4.4.2.4). This verifies that both cell types have the same ability to undergo trans-endothelial migration. The transmigration efficiency is calculated by taking cell counts of the normal wild-type neutrophil transmigrated on TNF-α treated endothelial cells (as the maximum (100%) transmigration competency marker) as the calculation reference number. The cell counts from each of the experiments were normalised to the reference numbers, and the percentages of transmigrated neutrophil populations were calculated accordingly (Figure 4.4.2.5). The TNF-α effect on neutrophil trans-endothelial migration ability was represented by the average percentage of transmigrated neutrophils, and after subtracting background numbers of the cell counts. The result showed that on average, 53% of the normal wild-type cells have successfully transmigrated after TNF-α treatment as compared to 4% exhibited by the homozygous calpain-1 KO cells (Figure 4.4.2.5). This result was shown to be significantly different and substantiated the effect of TNF-α on the normal wild-type and homozygous calpain-1 KO neutrophil's ability to transmigrate, and confirmed the qualitative outcomes observed under the microscope.

A) Normal Wild-type Neutrophils



B) Homozygous Calpain-1 KO Neutrophils

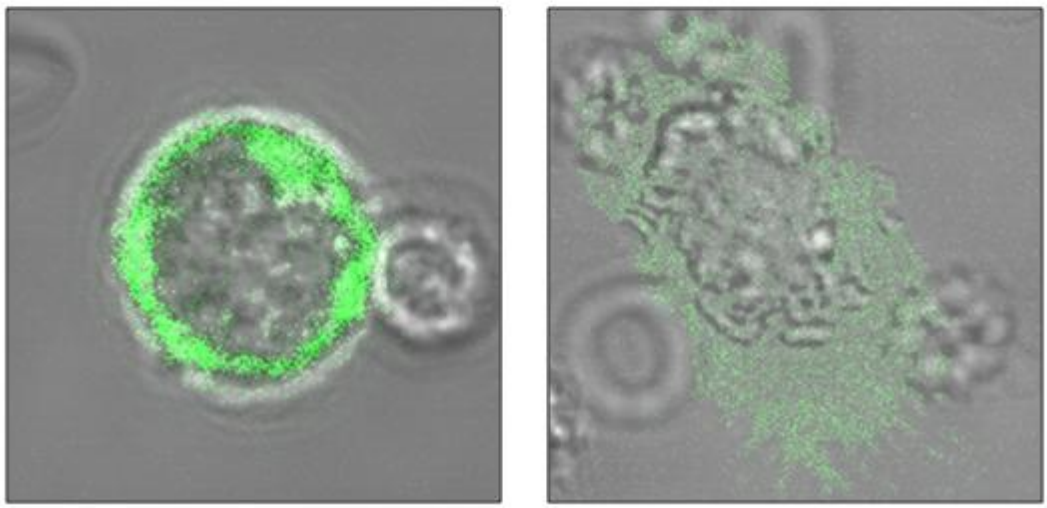


Figure 4.4.2.4: The immunofluorescent staining of integrins. The images above showed the immunofluorescent staining of integrins on the surface of (A) normal wild-type and (B) homozygous calpain-1 KO neutrophils during adhesion and spreading.

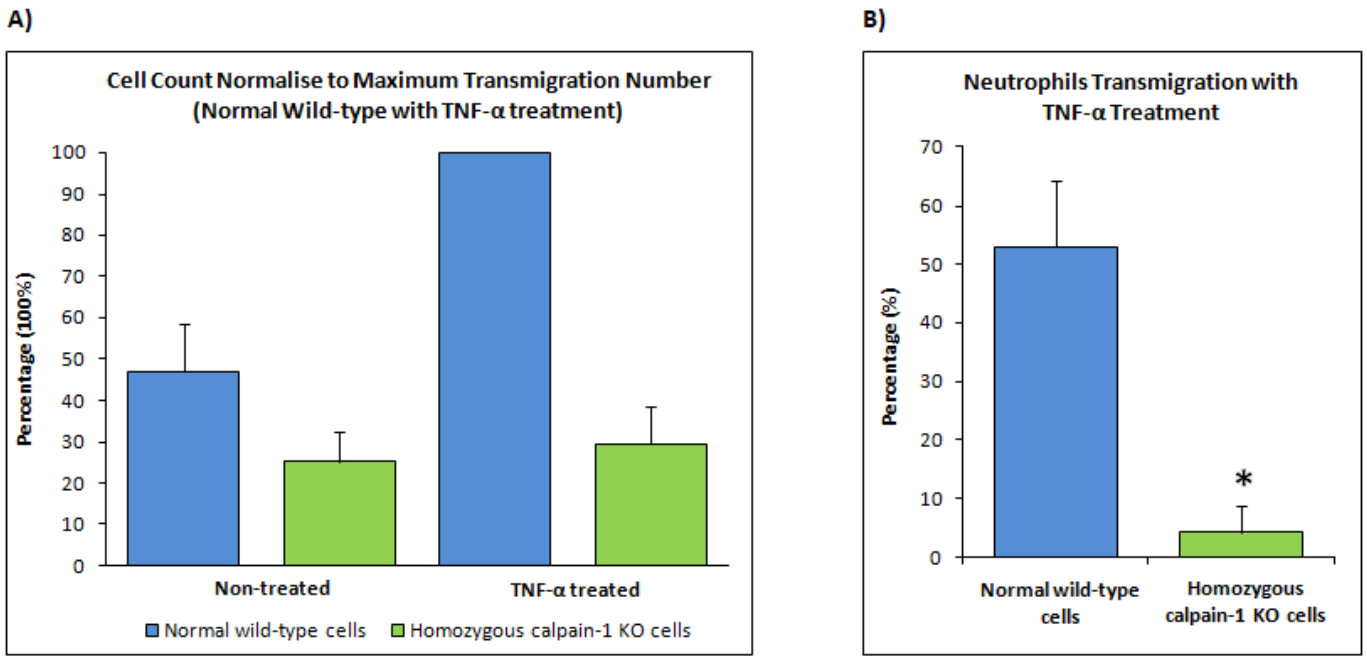


Figure 4.4.2.5: The normalised average population of transmigrated neutrophils against the normal wild-type cell count with TNF-α treatment. The graph in (A) represents normalised average populations of transmigrated neutrophils against the normal wild-type cell counts with TNF-α treatment (which is presented as 100%) as the maximum transmigration marker. Since every isolated neutrophil sample at the start of each experiment have different cell counts and that transmigrated normal wild-type neutrophil in TNF-α treated condition has shown to be the most proficient in the trans-endothelial migration (100% transmigration marker), all cell counts of other samples were calculated against the normal wild-type cells with TNF-α treatment in order to observe their differences. The result gave a contrasting graphical difference which suggests ineffectual transmigration ability shown by the homozygous calpain-1 KO cells. Graph (B) highlights the average difference between transmigrated neutrophil samples with TNF-α treatment after subtracting the background numbers of the normalised calculation. The result showed significant difference (p<0.05) between the normal wild-type and homozygous calpain-1 KO cell populations. Results are expressed as mean +/- S.E.M. and are representative of three separate experiments. *p<0.05 compared to the normal wild-type mice (analysis of variance followed by Student's t-test).

4.5. Discussion

The results in this chapter established that the absence of calpain-1 disrupted neutrophil's ability to transmigrate *in vitro* as well as their recruitment in the mice *in vivo*. The striking contrasts showed by the ability of normal wild-type neutrophils to transmigrate across the endothelial cells essentially confirmed the cellular signalling pathway involving TNF-α during the process. ICAM-1 is up-regulated following TNF-α treatment (Cinamon et al., 2004; Yang et al., 2005), and this possibly means that TNF-α subsequently sends intercellular signals along the ICAM-1 signalling pathway which would then interact with the neutrophil integrin molecules, and as a result promotes the trans-endothelial migration following the chemoattractant gradient. The possible cross-talk between ICAM-1 and integrin molecules due to the TNF-α treatment might have triggered the cellular mechanism in neutrophils that would then permit the cells to move without restraint before eventually transmigrating towards the chemoattractant.

However, TNF-α treatment did not show noticeable difference in the ability of homozygous calpain-1 neutrophils to transmigrate across the endothelial cells. The end results demonstrated that neutrophils from the homozygous calpain-1 KO mice completed transmigration with the same low numbers regardless of whether the endothelial cells were treated with TNF-α molecules or not. This suggested that without calpain-1, the ability of homozygous calpain-1 KO neutrophils to transmigrate across the endothelial cells has been disturbed. It implied the possibility of intracellular signalling transduction via the activation of ICAM-1 and its cross-linkage with neutrophil integrin molecules might not be followed up effectively in the absence of calpain-1.

Previous reports have indicated that the integrin molecules could function as signal transduction receptors as well as stimulate changes in the intracellular Ca^{2+} level which led to the generation of intracellular signalling pathways that would regulate cell behaviour and development (Sjaastad and Nelson, 1997). The cross-talk between both ICAM-1 and integrin molecules may cause upregulation of cellular components in neutrophils and simultaneously change the level of intracellular Ca^{2+}. Given the fact that calpain is a Ca^{2+}-activated protease, the rise in Ca^{2+} level would activates the enzyme in normal wild-type neutrophils before allowing the cells to perform their function accordingly. Engagement of the integrin during phagocytosis process (see Chapter 8) showed comparable Ca^{2+} signals between normal wild-type and homozygous calpain-1 KO neutrophils. This means that the defect in the trans-endothelial migration was not due to a Ca^{2+} signalling failing but was instead downstream of this. Thus, this result indicates that homozygous calpain-1 KO neutrophils did not undergo the trans-endothelial migration process as efficiently as normal wild-type neutrophils when the cells were put in a similar condition.

The findings from the *in vivo* study on the recruitment of neutrophils in normal wild-type and homozygous calpain-1 KO mice reiterate similar transmigration trend as recorded with the *in vitro* experiment. Female mice were randomly selected for this experiment as it has been reported that female mice are more susceptible to the inflammation development than the male (Hayashi et al., 2003; Melgert et al., 2005). The female mice challenged with airway allergen showed that they are more sensitive to the development of inflammatory reaction than male mice *in vivo*. It is established that the calpain-1 knocked-out effects may have a major influence on neutrophil recruitment into the peritoneal cavity after injecting the zymosan particles. Although it is shown that homozygous calpain-1 KO neutrophils have

transmigrated into the peritoneal cavity of the mice, the lower cell counts suggested that their ability to transmigrate towards the target site has been disrupted. This impairment may have happened at any point during the cell's progression right from their adhesion ability to their ability to undergo chemotaxis and phagocytosis. The *in vivo* effects indicated the absence of calpain-1 could have caused the defects at any one of the cellular steps during transmigration.

It is proposed that the Ca^{2+}-dependent signal produced via the integrin engagement would activate calpain-1 before cleaving the cellular tether and allowing the cells to perform their tasks. The degree of acute and chronic inflammation can be significantly attenuated by treating the collagen-induced arthritis in rats with calpain inhibitor I (Cuzzocrea et al., 2000). The calpain inhibitor I reduced neutrophil recruitment and eventually minimized the degree of tissue injury. A similar calpain inhibitory effect has also been shown in the transmigration ability of dendritic cells. Calpain inhibition in primary mouse dendritic cells leads to enhance accumulation of actin filaments which is associated with stabilisation of podosome turnover, and resulted in inefficient migration across the endothelial cells (Calle et al., 2006). This data is consistent with the role of calpain-1 in neutrophils. In conclusion, calpain-1 deficiency has caused the defect in the ability of neutrophils to undergo trans-endothelial migration *in vivo* and *in vitro*. A number of probable mechanisms could explain the disruption in trans-endothelial migration of calpain-1 deficient neutrophils, and one of the factors could be due to the difference in their surface morphology which provides the "membrane reservoir" for cell expansion.

Chapter 5

Calpain-1 Expression and Surface Morphology of Murine Neutrophils

5.1. Introduction

There were several possible mechanisms that could explain the defect in trans-endothelial migration of calpain-1 deficient neutrophils shown in Chapter 4. The effects will be explored in subsequent chapters. The subject of the work presented in this chapter provides the most obvious explanation, namely that calpain-1 deficiency has an influence on the morphology of neutrophils such that they are less able to transmigrate.

5.1.1. The Role of Calpain in Cell Surface Morphology

Calpain has been shown to have a major role in the intestinal microvilli brush border assembly during cell differentiation as well as disassembly in the case of bacterial invasions (Potter et al., 2003). By adding calpain inhibitor to the intestinal epithelial cell, the apical microvillus extension was reduced and then blocked the brush border assembly indicating calpain involvement in ezrin recruitments to the brush border microvilli (Potter et al., 2003). The tracheal pulmonary epithelium ciliogenesis with decreased calpastatin expressions demonstrated that the calpain inhibition resulted in partial reappearance of cilia in mice (Gomperts et al., 2004). Tracheal explants from the mice with decreased calpastatin showed that the cilia were not visible on the surface of the cells, but could in fact be detected on the tracheal explants after treatment with the calpain inhibitor. Although the two previous reports were for different types of cells, they both come to a rather conflicting idea on how calpain might be involved with the structural formation of the cells. Therefore, there is a possibility that knocking-out calpain-1 may have altered the surface morphology of calpain-1 deficient murine neutrophils.

5.2. Aims of the Chapter

The aims of the work in this chapter were to:

1. Differentiate the morphological characteristics on the surface of normal wild-type and homozygous calpain-1 KO neutrophils.
2. Analyse and compare the presence of microvilli on the surfaces of normal wild-type mouse with homozygous calpain-1 KO neutrophils.

The aims of this chapter will be achieved by detecting and identifying the presence of calpain-1 in fixed neutrophils from normal wild-type and homozygous calpain-1 KO mice by immunofluorescent staining. The morphological surfaces of neutrophils from the normal wild-type and homozygous calpain-1 KO mice will be examined using the scanning electron microscope (SEM).

5.3. Methods

This methodology section describes the protocol and technique used to perform the experimental work for this chapter. The methods include; detecting calpain-1 presence in normal wild-type and homozygous calpain-1 KO neutrophils through the immunofluorescent staining, and evaluating the microvilli or ridge structures on the surface of neutrophils taken from the mice.

5.3.1. Evaluating Wrinkle Coverage on Murine Neutrophils

Neutrophils isolated from normal wild-type and homozygous calpain-1 KO mice were kept in HBK medium. These cell samples were mixed in 2.5% (v/v) glutaraldehyde fixative solution and processed accordingly for observation under the SEM. The normal wild-type and homozygous calpain-1 KO neutrophils were identified, and pictures of the cells were taken. Morphological differences on each of the neutrophil pictures were then examined and analysed.

The appearance of wrinkles which cover the cell surface was analysed using Image J software (Figure 5.3.1.1). Assessment was made based on phase and contrast of the ridge structure shadows in each image of the cells from the SEM. First, the backgrounds of every individual 2-dimensional image of the neutrophils were blacked out. Then, the images were processed by filtering using the variance mode set at 10.0 pixels on the pictures. By selecting the binary mode, these images were processed again and made into binary profile images. These steps generated black and white images of the ridge structures on each cell. Finally, the processed images of the ridge structures that represent the wrinkles covering each cell was measured and analysed accordingly. The estimated percentages of the microvilli or “wrinkles” covering normal wild-type neutrophils were compared with neutrophils from the homozygous calpain-1 KO mice.

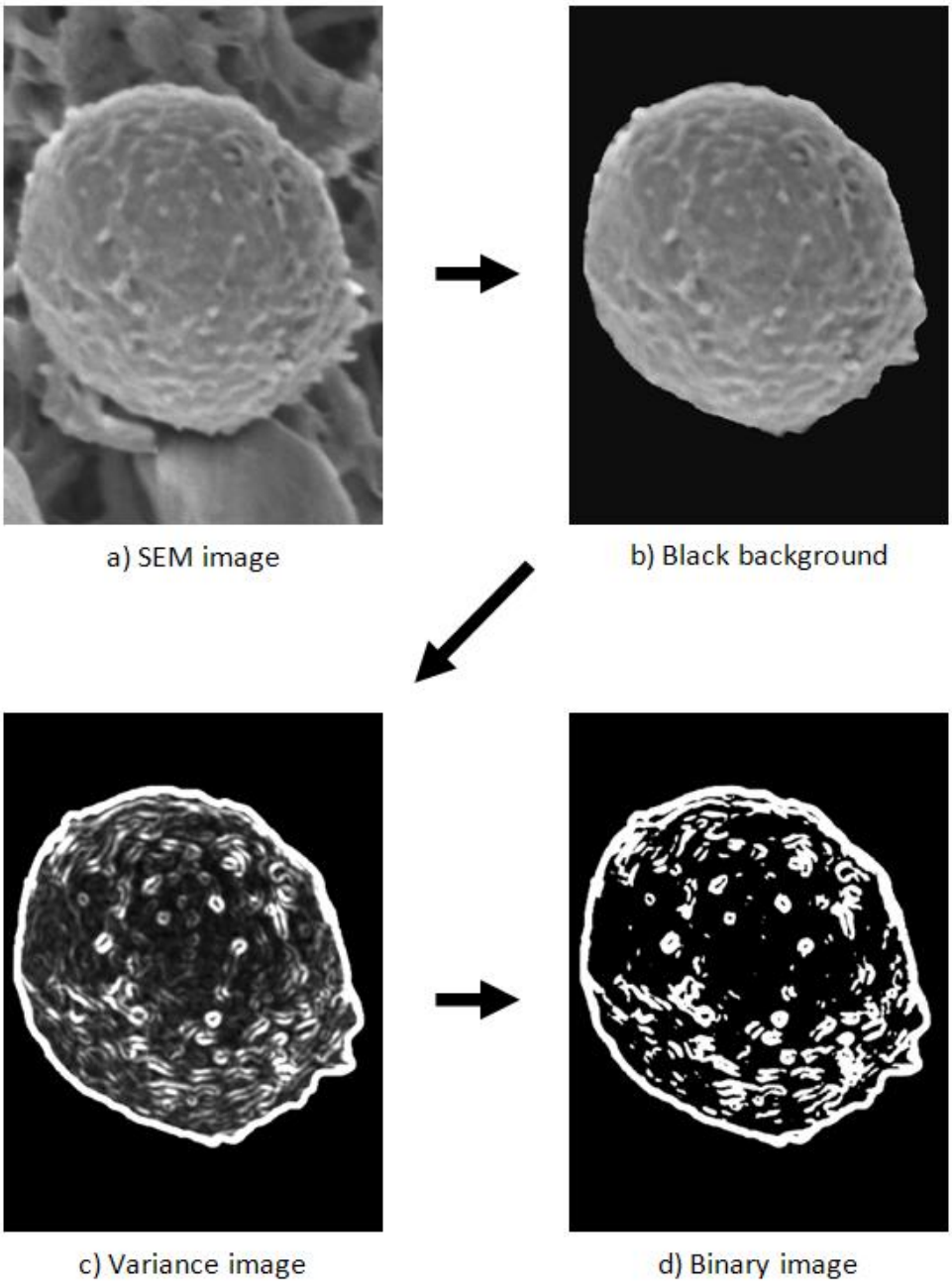


Figure 5.3.1.1: Neutrophils under the SEM before being analysed for wrinkle coverage. This figure shows pictures of neutrophils under the SEM before subsequently being processed using ImageJ software for the microvilli or “wrinkles” coverage analysis. Image in (a) shows the original neutrophil picture, before their background image is removed (b). The picture is then converted to a variance image (c), and finally sharpens and smoothens to a binary image (d), for the cell ”wrinkles” coverage analysis.

5.3.2. Calpain-1 Immunofluorescent Staining on Fixed Murine Neutrophils

This immunofluorescent calpain-1 staining work was prepared using a commercially available calpain-1 mouse monoclonal antibody (Santa Cruz Biotechnology) as the primary antibody and goat anti-mouse IgG conjugated to HRP (horseradish peroxidase) as secondary antibody. To begin with, isolated neutrophils were left on the glass coverslips to adhere for 10 to 15 minutes. The unbound cells were removed by washing twice with HBK medium. The cells were then fixed onto the coverslips by adding 100µl of 4% (w/v) formaldehyde and left for 10 minutes at room temperature. Next, the fixative solutions were removed by washing the cells 3 times with phosphate buffered saline (PBS). By adding 0.1% Triton solutions in PBS, the cells were permeabilized for 4 minutes at room temperature. The Triton solutions were subsequently removed by washing the cells twice with PBS.

In order to block non-specific binding of the antibodies, 100µl of 4% (v/v) horse serum diluted in PBS was added to the cells and left for 1 hour at room temperature. Before that, the primary antibody (mouse anti-calpain-1) was prepared by diluting it in PBS with 4% (v/v) horse serum in 1:100 µl ratios and then added to the cells to be left overnight at 4°C. Next, the primary antibody was washed 3 times with PBS (5 minutes for each wash). The anti-mouse IgG-FITC secondary antibody (1:100 µl antibody to PBS-4% horse serum ratio) was added and left in the dark for 1 hour at room temperature. Finally, the cells was washed twice with PBS (5 minutes for each wash) to remove any unbound secondary antibody. The immunofluorescence labelled neutrophils were monitored and the presence of calpain-1 was recorded with the inverted confocal laser scanning microscope using the fluorescein isothiocyanate (FITC) illumination setup.

5.4. Results

5.4.1. Wrinkle Coverage on Murine Neutrophils

The possible consequence of the calpain-1 knocked-out effect was examined by looking at the surface morphology of the neutrophils. The SEM images from both samples showed a typical ridge formation found on the surface of neutrophils. The “wrinkles” or microvilli that cover the surface of normal wild-type neutrophils appeared to have no distinctive differences with the homozygous calpain-1 KO cells. Pictures of neutrophils from both sets of samples taken with SEM showed no unique or general characteristics that separate the normal wild-type from the homozygous calpain-1 KO cells. Visibly, the surfaces of these neutrophils have the same unique ruffle appearance covering the cells. The quantitative analysis of wrinkles coverage on normal wild-type neutrophil is in the range of 20-30%, which is comparable to the 21-28% of wrinkle coverage on homozygous calpain-1 KO neutrophils (Figure 5.4.1.1).

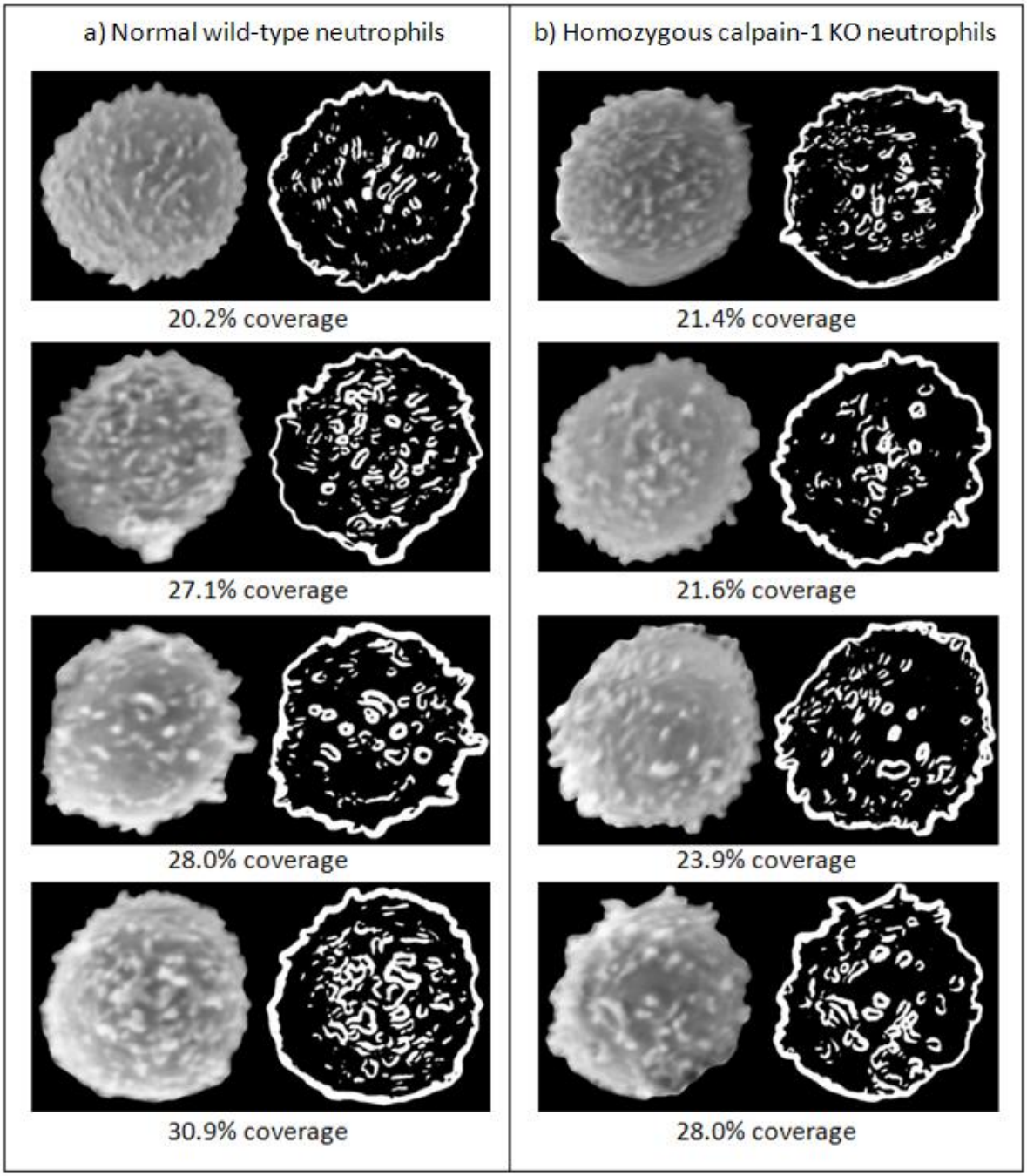


Figure 5.4.1.1: The evaluation of wrinkles coverage on the surface of neutrophils. These SEM and processed images represent the evaluation of "wrinkles" coverage on the surface of (A) normal wild-type and (B) homozygous calpain-1 KO neutrophils. As the results from the analysis show, the "wrinkles" or microvilli coverage for normal wild-type neutrophils is 20.2% to 30.9%, and for the homozygous calpain-1 KO cells it is from 21.4% to 28.0%. Both neutrophil samples give comparable results, which mean both of the cells have equal membrane "reservoirs" for expansion or spreading.

5.4.2. Calpain-1 Expression in Murine Neutrophils

There were very clear differences in the confocal microscopic images of calpain-1 immuno-stained normal wild-type and homozygous calpain-1 KO neutrophils. The staining of immunofluorescently labelled calpain-1 was prominent on normal wild-type neutrophils with calpain-1 distributed at the plasma membrane. The calpain-1 fluorescence signals can be detected in the majority of the cells in the population. In contrast, homozygous calpain-1 KO neutrophils failed to show any calpain-1 signals (Figure 5.4.1.1). Therefore, this finding confirms that calpain-1 gene deletion in the homozygous calpain-1 KO mice has resulted in a significant reduction in calpain-1 expression in neutrophils.

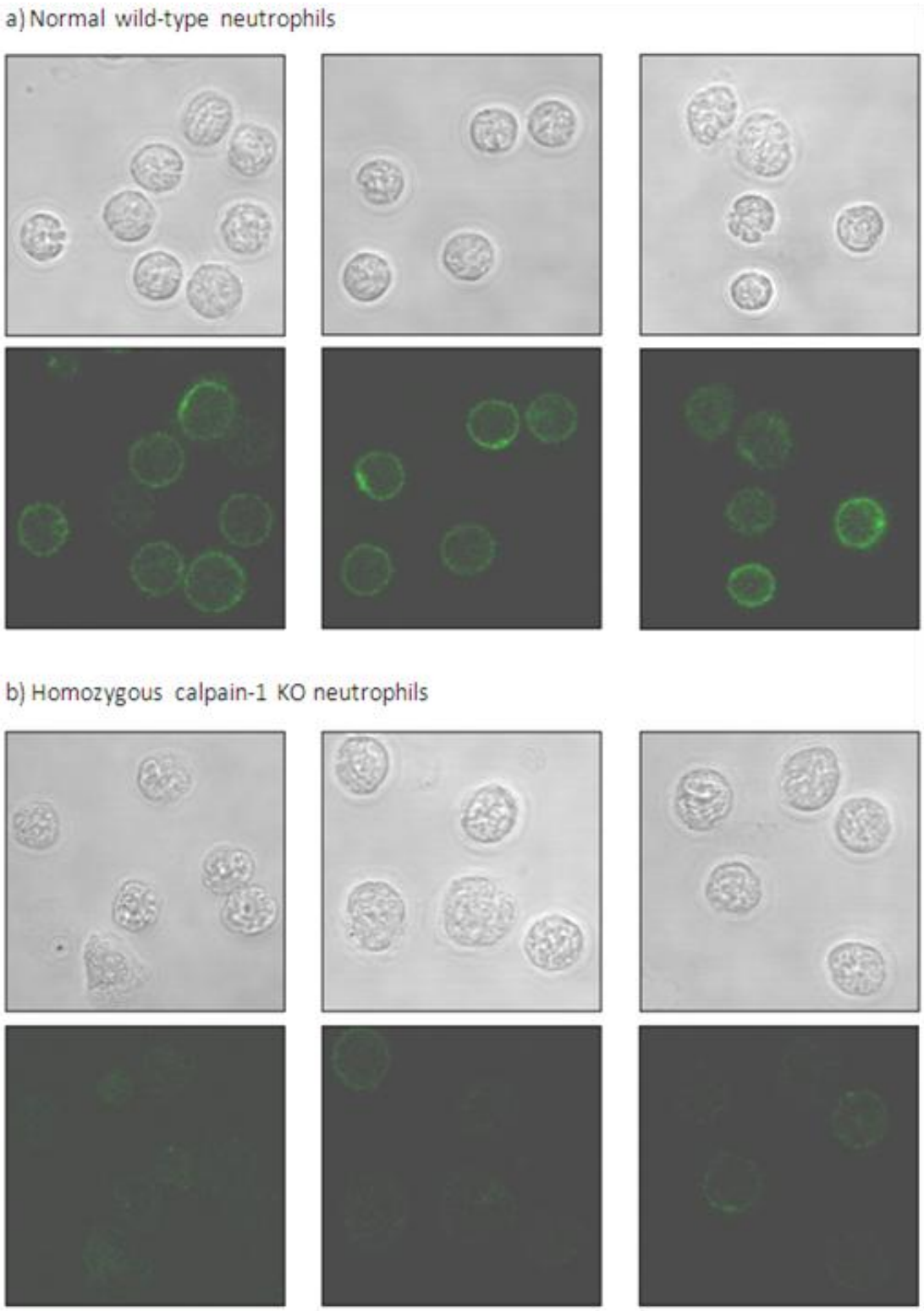


Figure 5.4.2.1: The calpain-1 immunofluorescence staining. The pictures above represent the immunofluorescence staining on (A) normal wild-type and (B) homozygous calpain-1 KO neutrophils under the confocal microscope. The normal wild-type cells displayed clear evidence of calpain-1 presence which showed strong signals lining the edge of the cell membranes, and as compared to the fluorescence staining on the homozygous calpain-1 KO cells which demonstrated only weak signals.

5.5. Discussion

The results on neutrophil cellular morphology showed that their surface structures were not affected by calpain-1 gene deletion. Although calpain might have different roles in cilia formation in the intestines (Potter et al. 2003) or in tracheal epithelial cells (Gomperts et al. 2004), it is illustrated here that calpain-1 was not involved in the typical formation of neutrophil “wrinkles”. Thus, it is suggested that by having the same amount of “wrinkles” covering their surfaces, both normal wild-type and homozygous calpain-1 KO neutrophils would have equal masses of “membrane reservoir” for cell expansions. The membrane “wrinkles” on the surface of neutrophils is lost as the cell spreads out (Dewitt and Hallett 2007). However, although both normal wild-type and homozygous calpain-1 KO neutrophils shared the same morphological appearance, their cellular activities may differ significantly.

Furthermore, the presence of the immunofluorescently labelled calpain-1 in normal wild-type neutrophils as opposed to the absence of calpain-1 in the homozygous calpain-1 KO neutrophils confirmed the knocked-out effect in the cells. Although it shows visual evidence of calpain-1 on the membranes of normal wild-type neutrophils, this does not indicate that calpain-1 is involved in regulating the shape changes of the cells. At this stage, calpain-1 functions in the cells are not fully understood. However, this immunofluorescent staining method provided the possibility of studying the role of calpain-1 in the regulation of neutrophil morphological changes. This technique will help to further understand the links between calpain-1 and cell spreading as well as in phagocytosis process, particularly in the enzyme translocations from the cytosolic region of the cells to their plasma membranes. In conclusion, knocking-out calpain-1 did not alter the microvilli or “wrinkles” formation on the

cellular surface of neutrophils. However, the possibility of defects shown by the homozygous calpain-1 KO neutrophils could be investigated by looking at their ability to spread as compared to the normal wild-type neutrophils.

Chapter 6

The Role of Calpain-1 in Cell Spreading

6.1. Introduction

Another possible contributory factor to the defect in the trans-endothelial migration ability observed in calpain-1 deficient neutrophils may be related to their ability to change shape and spread onto the surfaces. The data presented in this chapter seeks to address this possibility.

6.1.1. Neutrophil Adhesion and Spreading

The ability of neutrophils to adhere and spread marks the early stages of recruitment in the event of inflammation. Neutrophil infiltration requires the cells to attach onto the endothelial cells of the blood vessel and undergo spreading which changes their shape from spherical to a flattened form (Dewitt and Hallett, 2007). The capability to adhere and spread is important for neutrophils to complete their functions as the first line of defence against invading foreign particles. In view of that, it is essential to investigate the extent of calpain-1 involvement in neutrophil's ability to adhere and spread. Earlier studies have identified calpain-1 as one of the molecules that interacts with the cells cytoskeleton and plays a part in their spreading capacity (Potter et al., 1998).

By stably overexpressing the endogenous calpain inhibitor, calpastatin, and using the pharmacological inhibitors for calpain, the ability of fibroblasts to spread appeared to be inhibited (Potter et al., 1998). It is believed that calpain is involved in the mechanisms that caused actin remodelling during cell spreading. Although the functions have not been fully understood, the presence of several potential actin-associated substrates for calpain might

explain the actions performed by the enzyme (Ivetic and Ridley, 2004). This mechanism of action is believed to be involved in cytoskeletal reorganization process which is associated with the cell's plasma membrane and actin filaments. Reorganization of the cytoskeleton through calpain actions is thought to be the key step to release the cellular membrane binding before the cells are able to change shape and spread (Dewitt and Hallett, 2007).

As a Ca^{2+}-activated protease, calpain relies on the presence of Ca^{2+} to become active in order to perform its function. Intracellular Ca^{2+} storage release triggers a global Ca^{2+} influx across the cells and this can be triggered by "uncaging" IP_3 (inositol trisphosphate) within the cytosol (Hillson and Hallett, 2007). The elevated Ca^{2+} level through the IP_3 pathway causes a quicker rate of cell spreading (Dewitt and Hallett 2007). Thus, investigating the competency of neutrophils to adhere and spread, and together with their Ca^{2+} signalling could give a new insight on the extent of calpain-1 role in regulating neutrophil functions.

6.2. Aims of the Chapter

The aims of the work described in this chapter were to:

1. Compare the adhesion and spreading properties of circulating blood neutrophils from the normal wild-type with homozygous calpain-1 KO mice.
2. Determine spreading competency of individual neutrophils after fMLP stimulation and to compare their surface area of spreading measurements between the normal wild-type and homozygous calpain-1 KO cells.
3. Determine the spreading diameter of neutrophil populations from the normal wild-type and homozygous calpain-1 KO mice in a set time course.

4. Measure cytosolic Ca^{2+} levels through the IP_3 pathway and their effects in normal wild-type and homozygous calpain-1 KO cells.
5. Detect and determine the cellular locations of the calpain-1 enzyme in both normal wild-type and homozygous calpain-1 KO neutrophils during adhesion and spreading.

6.3. Methods

This methodology section describes the protocols and techniques used to perform the experimental works for this chapter. These methods include; the adhesion and spread area evaluation of individual neutrophils, measuring the diameter of neutrophil spreading in a population, and detecting calpain-1 expression in the normal wild-type and homozygous calpain-1 KO adherent neutrophil.

6.3.1. Adhesion and Spreading Evaluation of Individual Neutrophils

Neutrophils isolated from the blood of normal wild-type and homozygous calpain-1 KO mice were kept in suspension in HBK medium. Approximately 20µl of the suspended cells were put onto the glass coverslips on the confocal microscope heated stage (37°C) and allowed to settle down for 3 to 5 minutes. In order to determine cell adhesion, pictures of individual neutrophils were taken at the beginning of the experiments and their sizes were measured using the Leica Lite version of the confocal microscope software. After that, about 5µl of 1µM fMLP was added to the cell samples on the glass coverslips to examine their spreading capacity. About 10 minutes later, pictures of spread neutrophils from both normal wild-type and homozygous calpain-1 KO cell populations were documented and measured

accordingly. The identity of the cell was confirmed by adding 3 drops of 10% (w/v) acridine orange in HBK medium to stain their nuclei. This experiment was repeated on several isolated neutrophil samples and the graphs were plotted to detect disparity in the cell size. The data are reported as mean +/- S.E.M. The means from different experiments were compared by one-way analysis of variance. When significant differences were identified, the comparisons were subsequently made using the Student's *t*-test for unpaired values. Statistical significance was set at $p<0.05$.

6.3.2. Diameter Measurement of Spreading Neutrophil Populations

The spreading competencies of the whole neutrophil populations were performed in order to confirm the individual cell measurements. By using the Cellometer automated cell counter machine, the diameters of neutrophil populations were measured and calculated by setting a range of cell sizes from 9 to 20 micrometer. Minor modifications to the sample slides for the cell counter device were made by inserting glass coverslips in between the top and the bottom half of the slides (Figure 6.3.2.1). After that, 20µl of neutrophils (suspended in 500µl HBK medium) were pipetted onto the modified Cellometer slides and left to settle down for 2 minutes. Following that 5µl of 1µM fMLP was introduced to the cells to stimulate spreading. The spreading diameters of the cells in the populations were monitored over 15 minutes. Data and images of the normal wild-type and homozygous calpain-1 KO neutophils spreading were recorded at 1, 3, 5, 7, 9, 11, 13 and 15 minutes. The data are reported as mean +/- S.E.M. The means from different experiments were compared by one-way analysis of variance. The comparisons were made using Student's *t*-test for unpaired values and the statistical significance set at $p<0.05$.

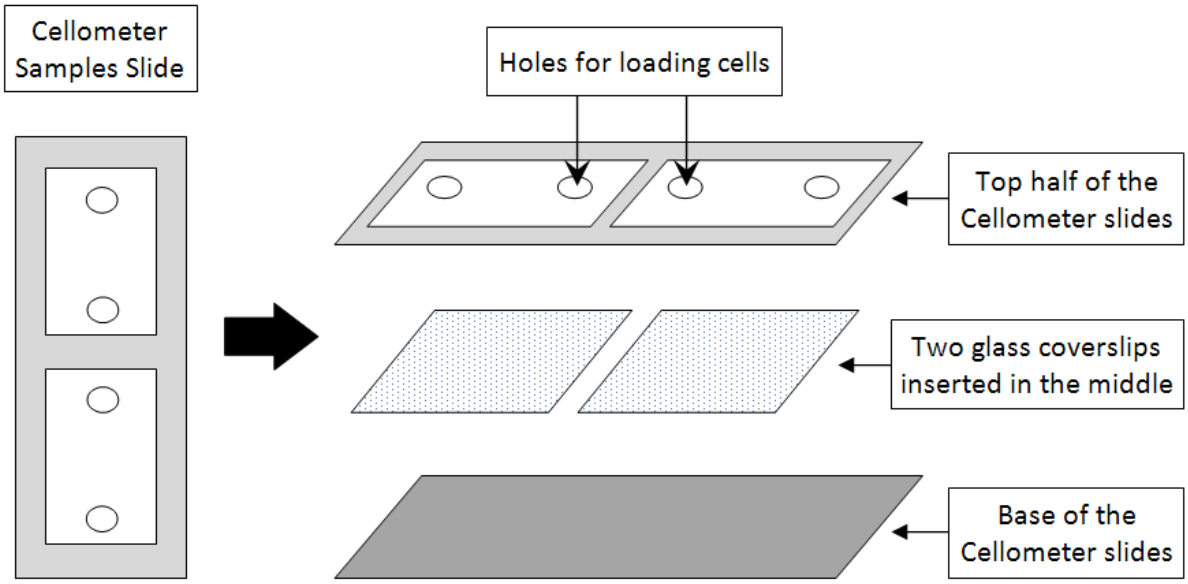


Figure 6.3.2.1: The Cellometer slides and glass coverslips for neutrophil spreading. The Cellometer slides were taken apart and glass coverslips were inserted as the surface for neutrophils to adhere and spread. The slides were then attached back together.

6.3.3. Ca^{2+} Signalling and IP_3 Uncaging

The changes in cytosolic Ca^{2+} levels in this experiment were measured by adding the propionyloxymethyl (PM)-caged IP_3 (Enzo® Life Sciences) to the normal wild-type and homozygous calpain-1 KO neutrophils. The caged IP_3 derivative was disolved in the supplied dry DMSO and Pluronic F127, before mixing with cells in 1:1000 ratio in Eppendorf tubes to a final concentration in the 1-2µM range. In order to avoid the accidental activation of the caged IP_3, this step has to be done in the dark before the suspended cells were wrapped in aluminium foil and left at room temperature for 20 minutes. After that, 1µl of Fluo-4 AM (Invitrogen Ltd) was added to the cell suspension and left in the dark at room temperatures for another 20 minutes. Before commencing the experiment, the cells loaded with caged IP_3 and Fluo-4 were put onto the glass coverslips and left to adhere for 5 minutes. Individual cells were then exposed to the UV light at 360nm wavelength for 10 seconds. The changes in cytosolic Ca^{2+} levels and the cells responses in normal wild-type and homozygous calpain-1 KO neutrophils were recorded using confocal laser scanning microscope. The graphs for Ca^{2+} signals were plotted and analysed in relation to the cell reactions with regard to the changes in cytosolic Ca^{2+} level.

6.3.4. Calpain-1 Immunofluorescent Staining on Adhered Neutrophils

This immunofluorescent calpain-1 staining work was prepared using a commercially available calpain-1 mouse monoclonal antibody (Santa Cruz Biotechnology) as the primary antibody and goat anti-mouse IgG conjugated to FITC as secondary antibody. To begin with, isolated neutrophils were left on the glass coverslips to adhere for 10 to 15 minutes. The

unbound cells were removed by washing twice with the HBK medium. The cells were then fixed by adding 100µl of 4% (w/v) formaldehyde and left for about 10 minutes at room temperature. The fixative solutions were removed by washing the cells 3 times with PBS. By adding 0.1% (v/v) Triton X-100 solution in PBS, the cells were permeabilized for 4 minutes at room temperature. The Triton solutions were subsequently removed by washing the cells twice with PBS.

In order to block non-specific binding of the antibodies, 100µl of 4% (v/v) horse serum diluted in PBS was added to the cells and left for 1 hour at room temperature. Calpain-1 as the primary antibody was prepared by diluting it in PBS with 4% (v/v) horse serum in a 1:100 µl ratio which was then added to the cells to be left overnight at 4°C. Subsequently, the primary antibody was washed 3 times with PBS solution (5 minutes for each wash). The secondary antibody (in 1:100 µl antibody to PBS-4% horse serum ratio) was then added before the slides were left in the dark for 1 hour at room temperature. Finally, the slide was washed twice with PBS (for 5 minutes with each wash) to remove any unbound secondary antibodies. The location of calpain-1 immunofluorescence signals in both normal wild-type and homozygous calpain-1 KO neutrophils were observed and distinguished using the inverted confocal microscope.

6.4. Results

6.4.1. Measurements of Individual Neutrophil Spreading Diameters

Neutrophils isolated from mouse blood samples were distinguished from the other white blood cells (WBCs) using AO to stain their nuclei. In addition, adhered (non-spread) WBCs and neutrophils could also be classified according to their sizes (Figure 6.4.1.1a). The areas of spreading for neutrophils from the cell samples were analysed using mathematical formula for measuring the ellipse area (Pierce 2011) as follow: Ellipse area = π x a x b (Figure 6.4.1.1b). After stimulation with fMLP, neutrophil spreading of both normal wild-type and homozygous calpain-1 KO mouse derived cells were evaluated by drawing X and Y axis for each of the cells in order to measure their radii (Figure 6.4.1.2).

The areas of neutrophil spreading were plotted in the X and Y scattered graphs. The graphs illustrated the raw data of surface area measurement for normal wild-type and homozygous calpain-1 KO neutrophils. The graphs showed that the range of surface area measurements of homozygous calpain-1 KO neutrophils is smaller than normal wild-type neutrophils to some extent (Figure 6.4.1.3). This is represented by the plotted measurement of homozygous calpain-1 KO neutrophils with a lower range of surface area (plotted dots are mostly placed closer to the X axis); as compared to the range of surface area of normal wild-type neutrophils (the dots are plotted slightly further from the X axis). This indicates that the normal wild-type neutrophils have a fairly larger surface area when spreading, as compared to the homozygous calpain-1 KO neutrophils. This experiment was repeated on different batches of neutrophils and consistently showed similar results. Thus, this finding indicates that the calpain-1 gene deletion has interfered with neutrophils ability to spread.

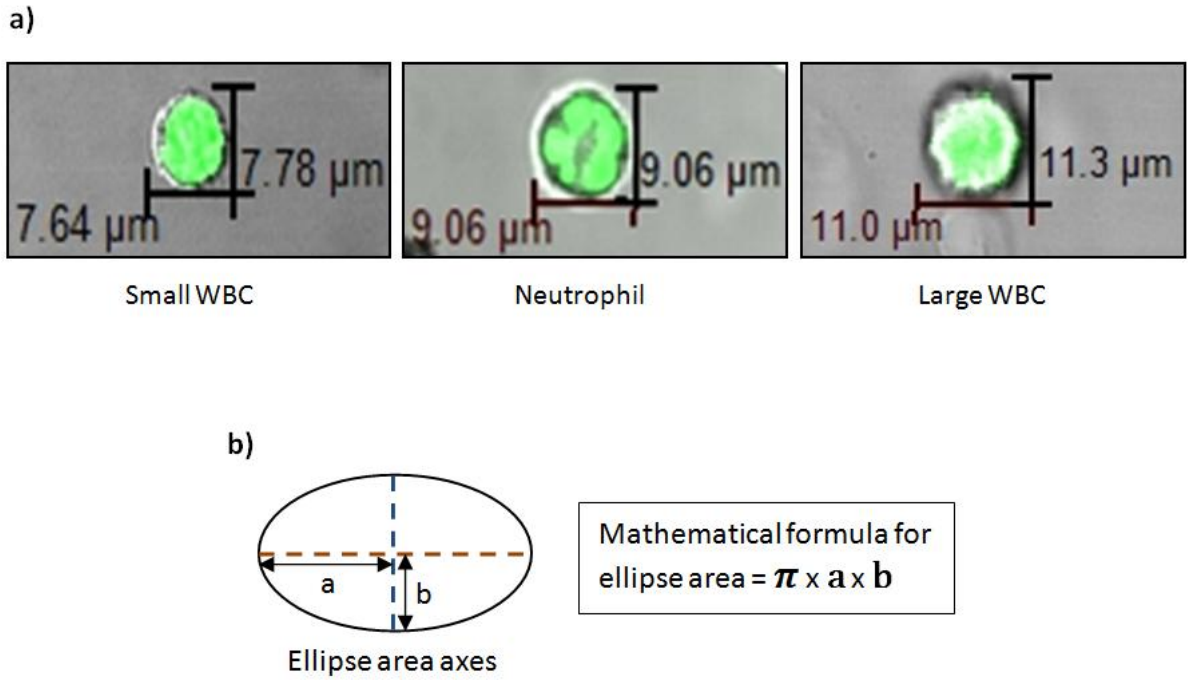


Figure 6.4.1.1: Pictures of neutrophils and other WBCs in the cell samples. The pictures in (a) represents neutrophils and other WBCs in the cell samples which are distinguished by their differences in size and nucleus stains. The illustration in (b) shows the ellipse area and its axes for measurement using the mathematical formula.

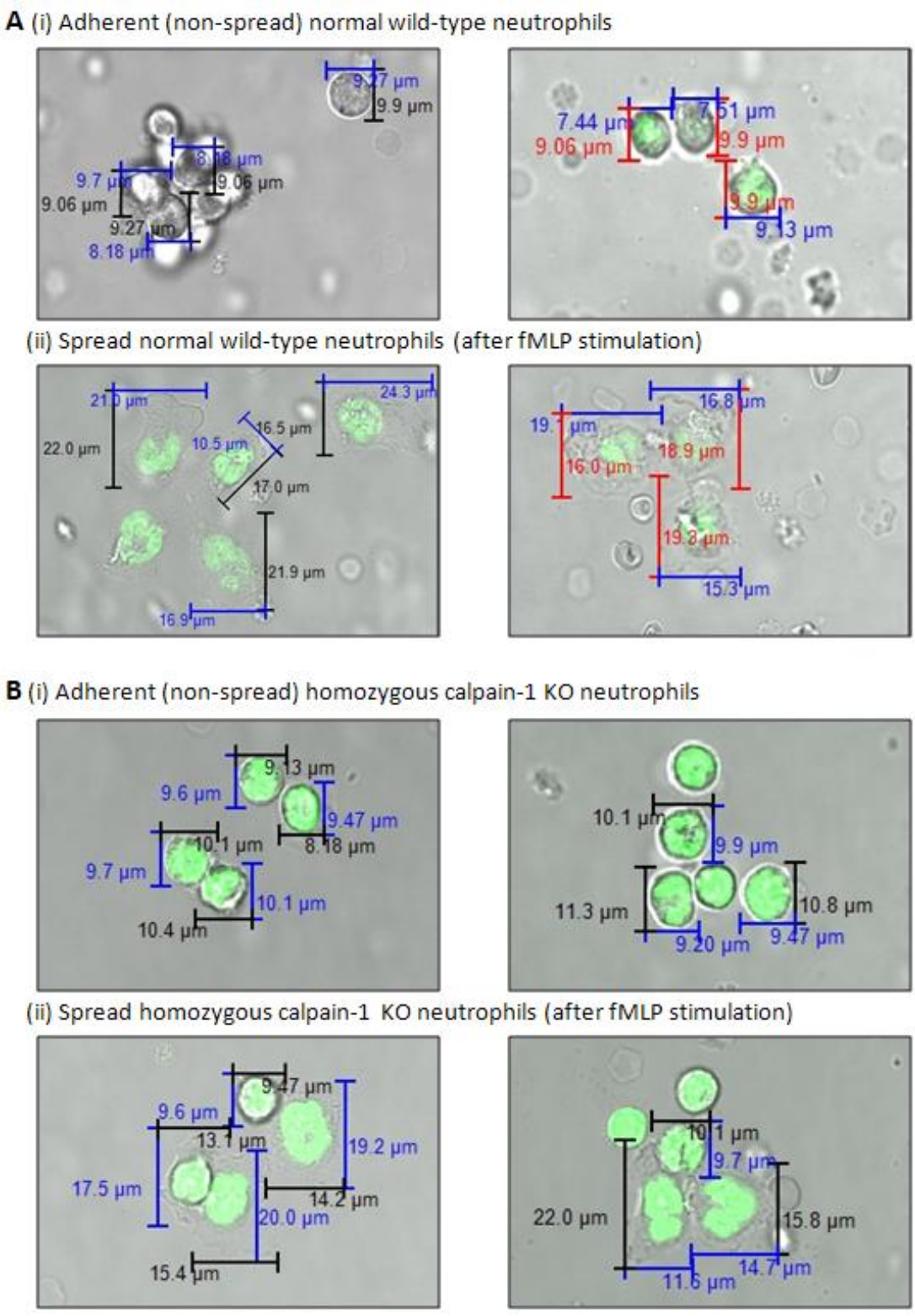


Figure 6.4.1.2: The sizes of neutrophils adhered onto the glass coverslips. The images in A(i) represent the sizes of neutrophils adhered onto the glass coverslips from normal wild-type mice and their spreading sizes as illustrated in A(ii) after adding fMLP. The pictures in B(i) showed the adhered homozygous calpain-1 KO neutrophil sizes before spreading and after the fMLP stimuli, B(ii). The acridine orange stains exhibits the nucleus of adhered and spread neutrophils.

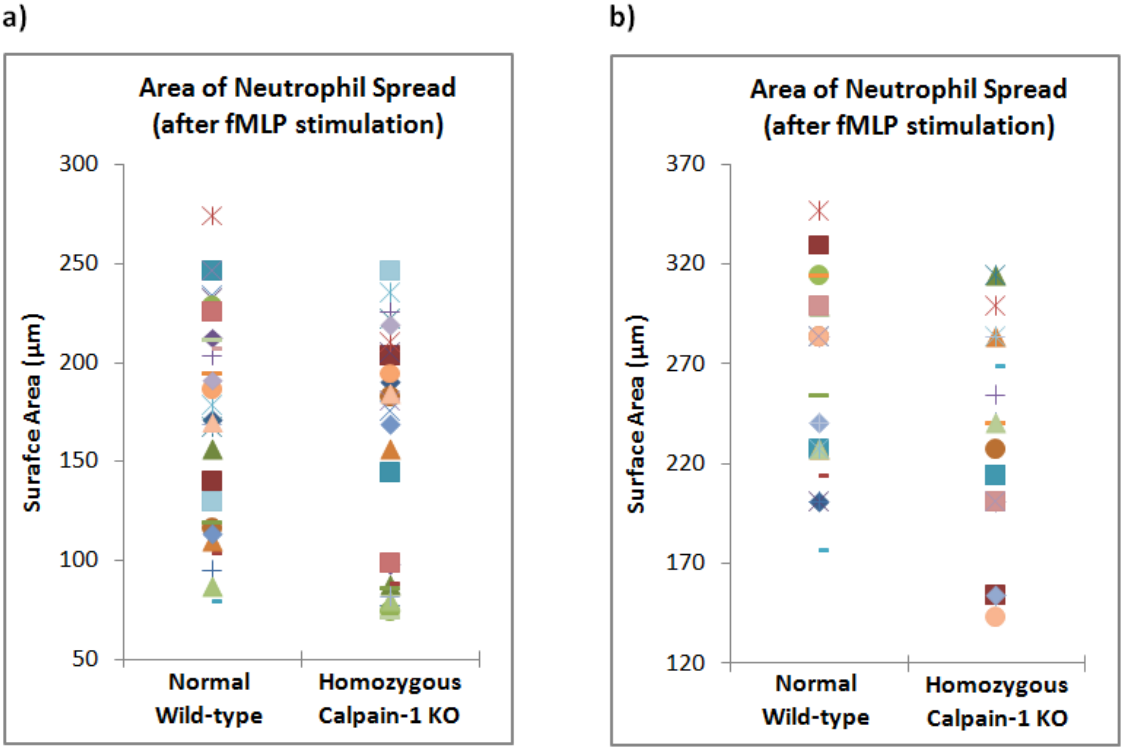


Figure 6.4.1.3: The raw graphical data representing the neutrophil surface area of spreading. The graphs in (a) and (b) represent data from two different experiments of normal wild-type and homozygous calpain-1 KO neutrophil measurement of their surface area of spreading. The range of homozygous calpain-1 KO neutrophil surface area is somewhat smaller than the normal wild-type neutrophil measurements. This is indicated by the plotted measurement of homozygous calpain-1 KO neutrophils with a lower range of surface area (plotted dots are mostly placed closer to the X axis); as compared to the range of surface area of normal wild-type neutrophils (the dots are plotted slightly further from the X axis).

6.4.2. Diameter Measurement of Spread Neutrophil Populations

In order to follow the spreading process of a number of neutrophils in actual time, a second approach was adopted. By setting a range of diameter for cell measurements with the Cellometer cell counter apparatus and software, the progression of the cell spreading on the slides was recorded and images taken at designated time points (Figure 6.4.2.1). The data verified that the diameter of neutrophils from normal wild-type cell populations measured at the range of more than 9µm, 11µm, 13µm and 15µm have a higher percentage compared to the homozygous calpain-1 KO cells (Figure 6.4.2.2).

Neutrophils from the normal wild-type mice showed a dramatic increase in cell size after 1 minute of fMLP introduction and continued to expand until they have reached the maximum after about 5 or 7 minutes. As for the cell populations from homozygous calpain-1 KO mouse, the diameter of these cells reached their biggest spreading size after the first minute and then remained significantly smaller as compared to the normal wild-type cells samples throughout 15 minute period of the experiments. Therefore, the results from this work concurred with the data gathered from the experiments of single cells spreading on glass coverslips and gave further insight of the cells ability to spread after being introduced to fMLP.

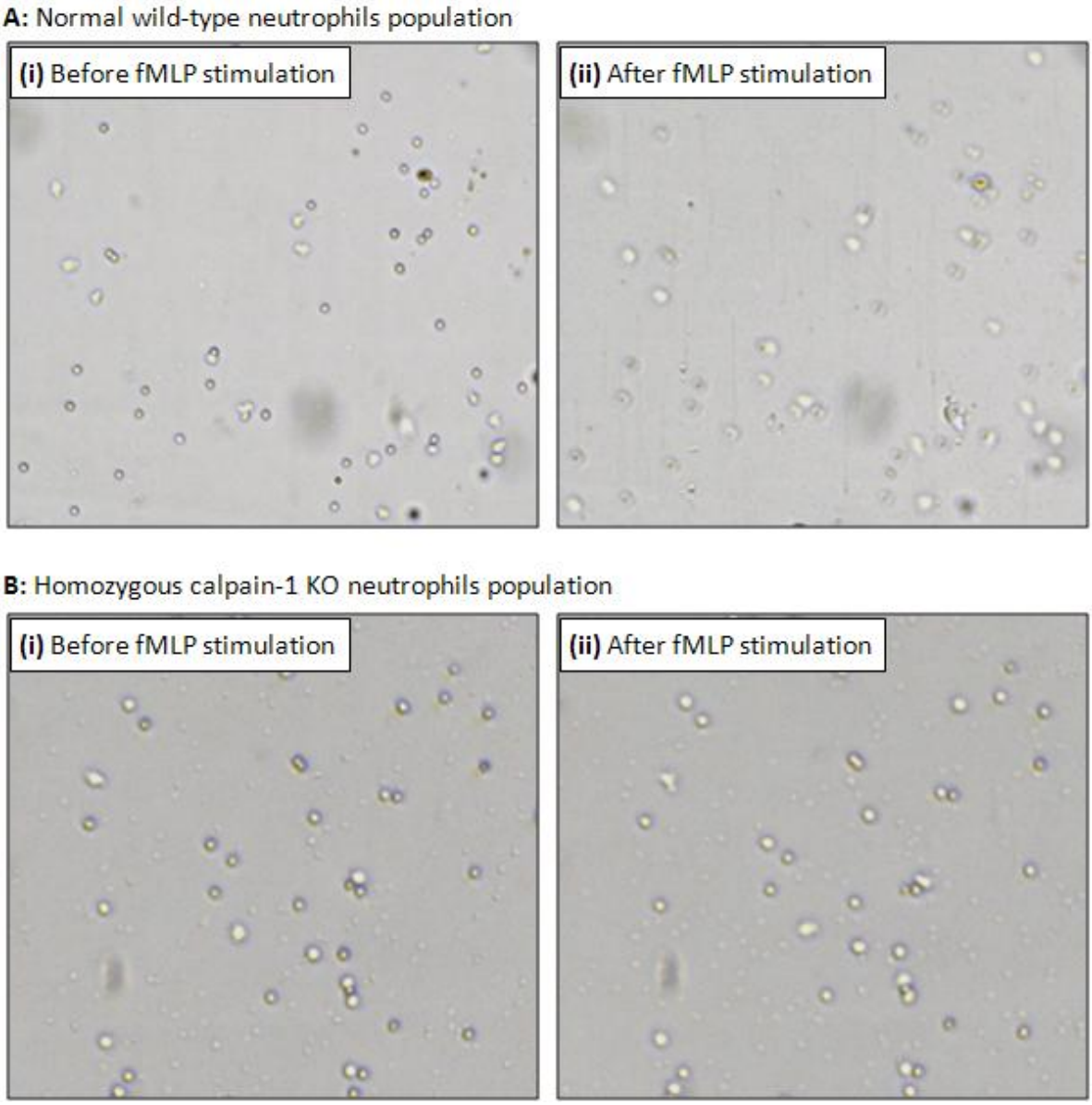


Figure 6.4.2.1: Pictures of neutrophils at the start and at the end of fMLP spreading experiments. The pictures in A(i) are the neutrophil populations from normal wild-type mice taken at the start of the experiments before fMLP was added. The cell sizes markedly grew bigger at the end of the 15 minutes period A(ii) after being stimulated by fMLP. The images in B(i) and B(ii) are from homozygous calpain-1 KO neutrophil populations captured at the beginning and at 15 minutes after fMLP was added respectively. In order to determine spreading differences between normal wild-type and homozygous calpain-1 KO neutrophil populations, the measurements of the cells spreading diameter were plotted into graphical figures.

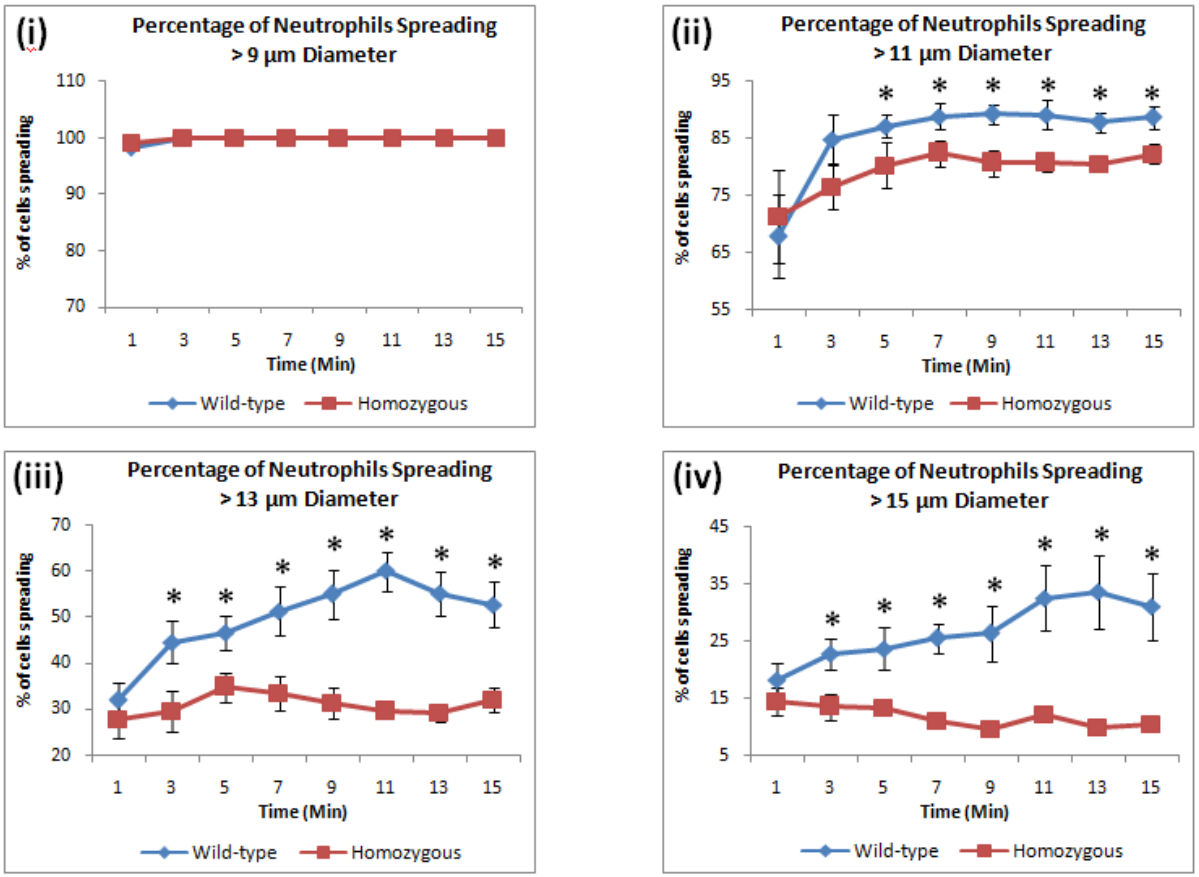


Figure 6.4.2.2: The graphs of neutrophil spreading diameters. The graphs above compare the neutrophil spreading diameters of normal wild-type and homozygous calpain-1 KO cell populations. Spreading diameters of neutrophils measured at 11, 13 and 15 µm (graph ii, iii and iv respectively) showed significant differences between them, as the average percentage of normal wild-type cells spreading diameters recorded in the 15 minute period were greater than homozygous calpain-1 KO neutrophils. The average spreading measurements from three different experiments were compared by one-way analysis of variance. The comparisons were made using Student's *t*-test for unpaired values and the statistical significance set at $p<0.05$.

6.4.3. Rise in Cytosolic Ca^{2+} Level via IP_3 Uncaging

One possible explanation for the ineffectual spreading ability of calpain-1 deficient neutrophils may b due to the dysfunction in Ca^{2+} signalling. The cytosolic Ca^{2+} level upon IP_3 uncaging in neutrophils from the normal wild-type and homozygous calpain-1 KO mice were therefore measured. The results show that the majority of normal wild-type cells (26/30 cells) were capable of generating Ca^{2+} signal via the IP_3 signalling pathway. The cells showed a single peak of cytosolic Ca^{2+} rise, which was not sufficient to trigger cell spreading (Figure 6.4.3.1). However, a few of the cells (4/30 cells) had multiple Ca^{2+} peaks that subsequently prompted the cells to spread (Figure 6.4.3.2). A similar outcome was seen with homozygous calpain-1 KO cells. Most of these cells (25/30 cells) showed that there is an increase in their cytosolic Ca^{2+} level with a single peak recorded, but this was not enough to induce the cells to spread (Figure 6.4.3.3). As with normal cells some of these homozygous calpain-1 KO cells (5/30 cells) showed multiple cytosolic Ca^{2+} peaks and spreading (Figure 6.4.3.4).

The results proved that the homozygous calpain-1 KO cells were capable of signalling Ca^{2+} through the IP_3 signalling pathway. This indicates that the absence of calpain-1 did not disrupt the ability to release the intracellular Ca^{2+} store. In addition, the results also mean that uncaging the IP_3 alone was not sufficient to elevate cytosolic Ca^{2+} to a level which could stimulate spreading in either of the normal wild-type or calpain-1 deficient cells. However, there were signs that neutrophils of both samples with a single Ca^{2+} peak expanded slightly after the IP_3 uncaging. Therefore, this signifies that the normal wild-type and homozygous calpain-1 KO neutrophils cells responded in a similar manner following the uncaging of IP_3.

Normal Wild-type Neutrophils

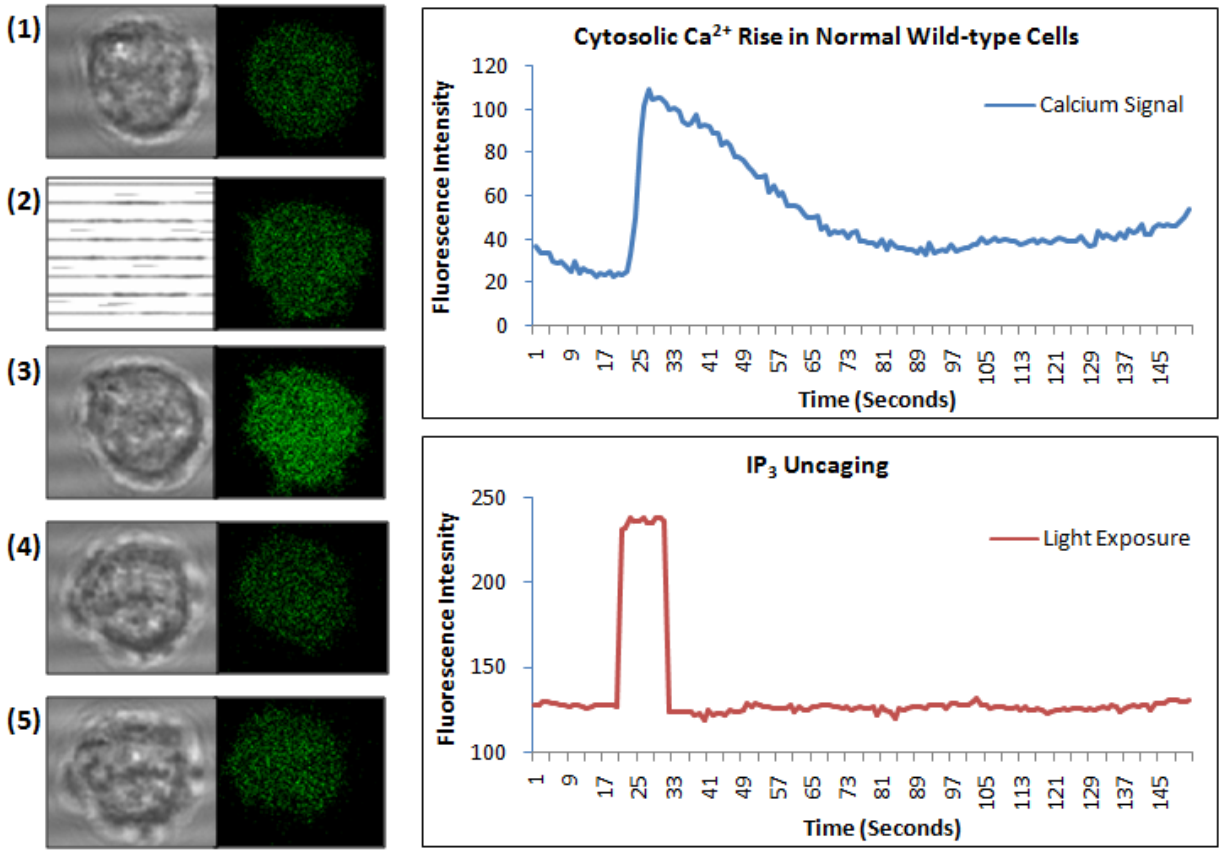


Figure 6.4.3.1: Images of normal wild-type neutrophils upon IP_3 uncaging. The greyscale and fluorescence images in the sequence above (1-5) represent the normal wild-type cells upon IP_3 uncaging. The graphs represent the cytosolic Ca^{2+} signalling in the cell and the moment the IP_3 is uncaged. Picture (1) is the cell at the start of the experiment and (2) the moment IP_3 is uncaged by UV light exposure. Picture in (3) represents the peak of cytosolic Ca^{2+} level before it descends (4). The greyscale picture in (5) demonstrated that the cell has expanded slightly but did not cause full spreading after the IP_3 uncaging (26/30 cells).

Normal Wild-type Neutrophils

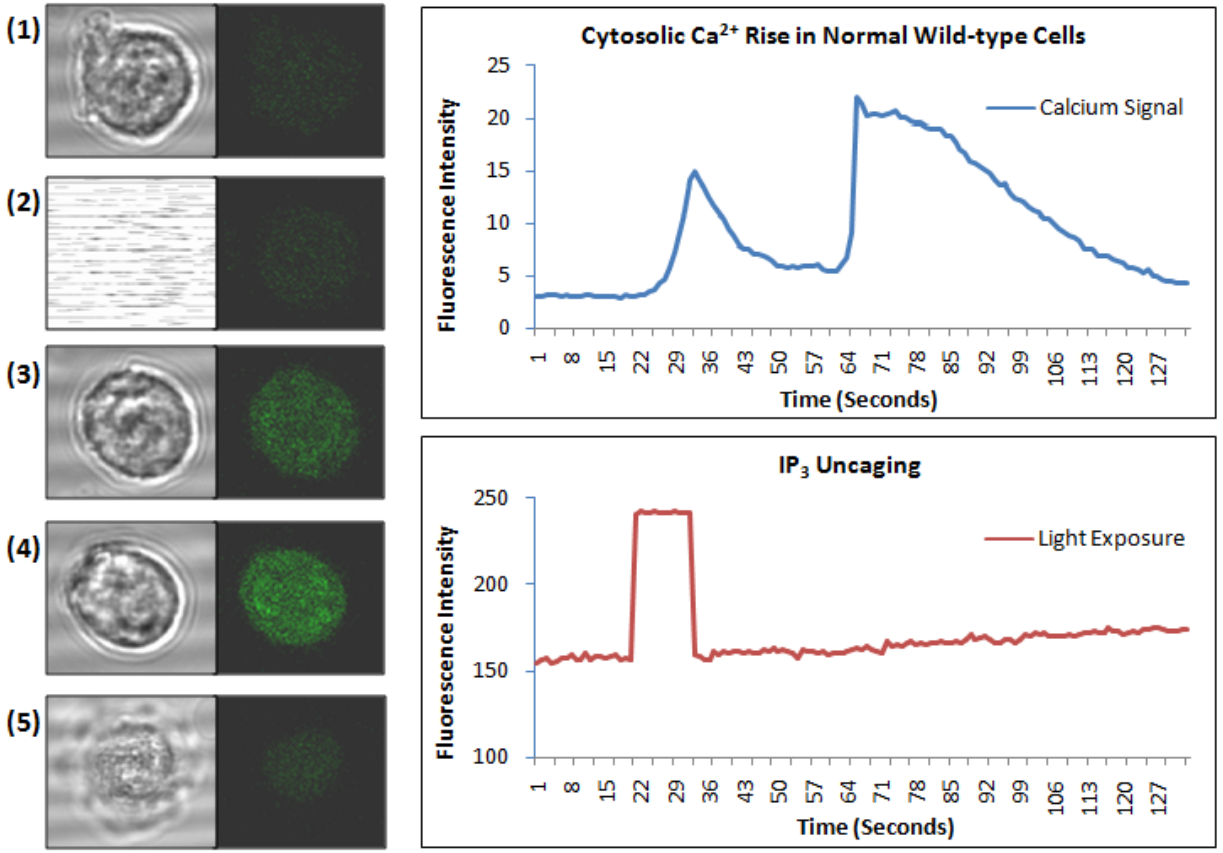


Figure 6.4.3.2: Image representing the normal wild-type cell Ca^{2+} signal and spreading upon IP_3 uncaging. This sequence of greyscale and fluorescence images from (1) to (5) represents the normal wild-type cell upon IP_3 uncaging. The graphs show the cytosolic Ca^{2+} signalling in the cell and the moment the IP_3 is uncaged. The picture in (1) is the cell at the start followed by the moment IP_3 is uncaged by UV light exposure (2). The pictures in (3) and (4) correspond to the two peaks of cytosolic Ca^{2+} level respectively. The greyscale picture in (5) showed that the cell has fully spread following the multi-peak of Ca^{2+} signals after the IP_3 uncaging (4/30 cells).

Homozygous Calpain-1 KO Neutrophils

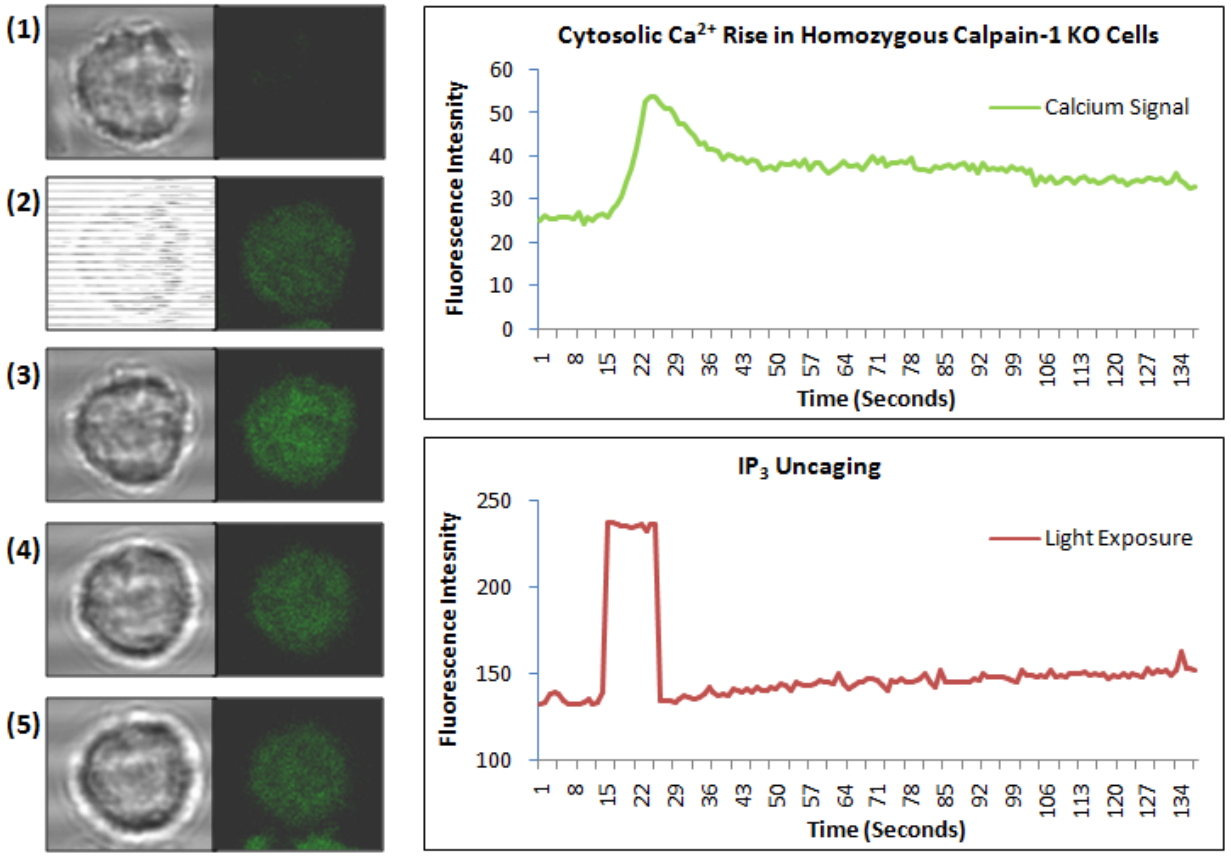


Figure 6.4.3.3: Series of images of homozygous calpain-1 KO cells Ca^{2+} signal upon IP_3 uncaging. The series of greyscale and fluorescence images above (1-5) represent the homozygous calpain-1 KO cells upon IP_3 uncaging. The graphs represent the cytosolic Ca^{2+} signalling and the moment IP_3 is uncaged. Picture (1) is the cell at the start of the experiment and the instant IP_3 is uncaged by the UV light exposure (2). The picture in (3) is the moment when cytosolic Ca^{2+} level reached its peak before descending (pictures 4 and 5). The greyscale picture in (5) showed that the cell has expanded slightly with the single Ca^{2+} rise but failed to cause spreading (25/30 cells).

Homozygous Calpain-1 KO Neutrophils

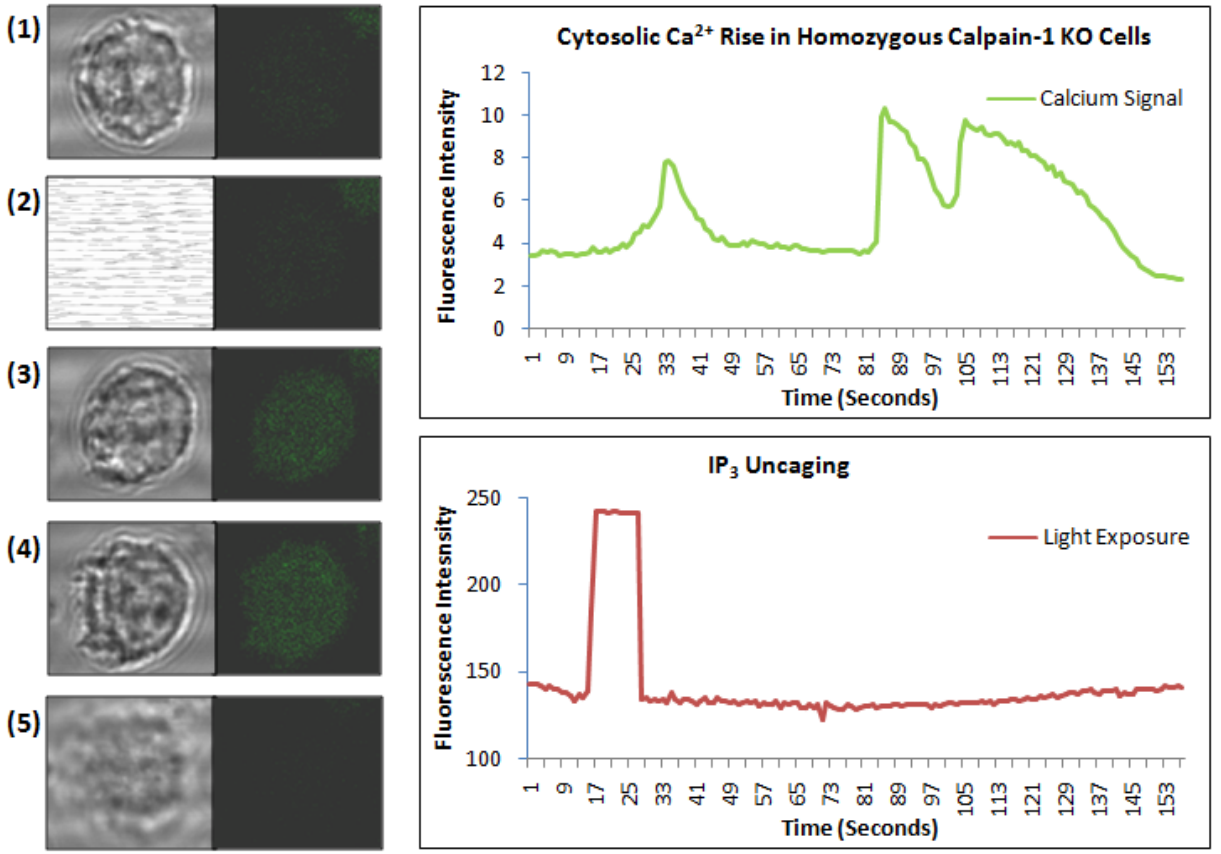


Figure 6.4.3.4: Images in series of homozygous calpain-1 KO cells Ca^{2+} signal and spreading upon IP_3 uncaging.The greyscale and fluorescence images in the series above (from 1 to 5) represent the homozygous calpain-1 KO cells upon IP_3 uncaging. The graphs indicate the cytosolic Ca^{2+} signalling in the cells and the instant IP_3 is uncaged. The picture in (1) is the cell at the start of experiment followed by the moment IP_3 is uncaged by UV light exposure (2). Pictures (3) and (4) represent the first and second cytosolic Ca^{2+} peak respectively. The greyscale picture in (5) showed that the cell has spread, following multiple Ca^{2+} peaks after the IP_3 uncaging (5/30 cells).

6.4.4. Calpain-1 Location in Adhered Neutrophil

The images of calpain-1 immunofluorescence staining in the fixed normal wild-type and homozygous calpain-1 KO neutrophils spreading on the glass coverslips showed a striking result. In normal wild-type cells, the fluorescence signals were prominent on the cells which have properly adhered and ready for spreading (Figure 6.4.4.1a). In these cells, the fluorescence signals for calpain-1 were predominantly high at the edge of the cells (Figure 6.4.4.1a). This means that calpain-1 was located on the plasma membrane of cells which were about to spread. The calpain-1 fluorescence stains on the cells that had spread were rather difficult to be detected. In contrast, there was no fluorescence staining on the homozygous calpain-1 KO cells, except for a weak and barely visible fluorescence which was uniform at the plasma membrane and cytosol (Figure 6.4.4.1b). The immunofluorescence staining confirmed the calpain-1 knock-out effect on homozygous calpain-1 KO neutrophils.

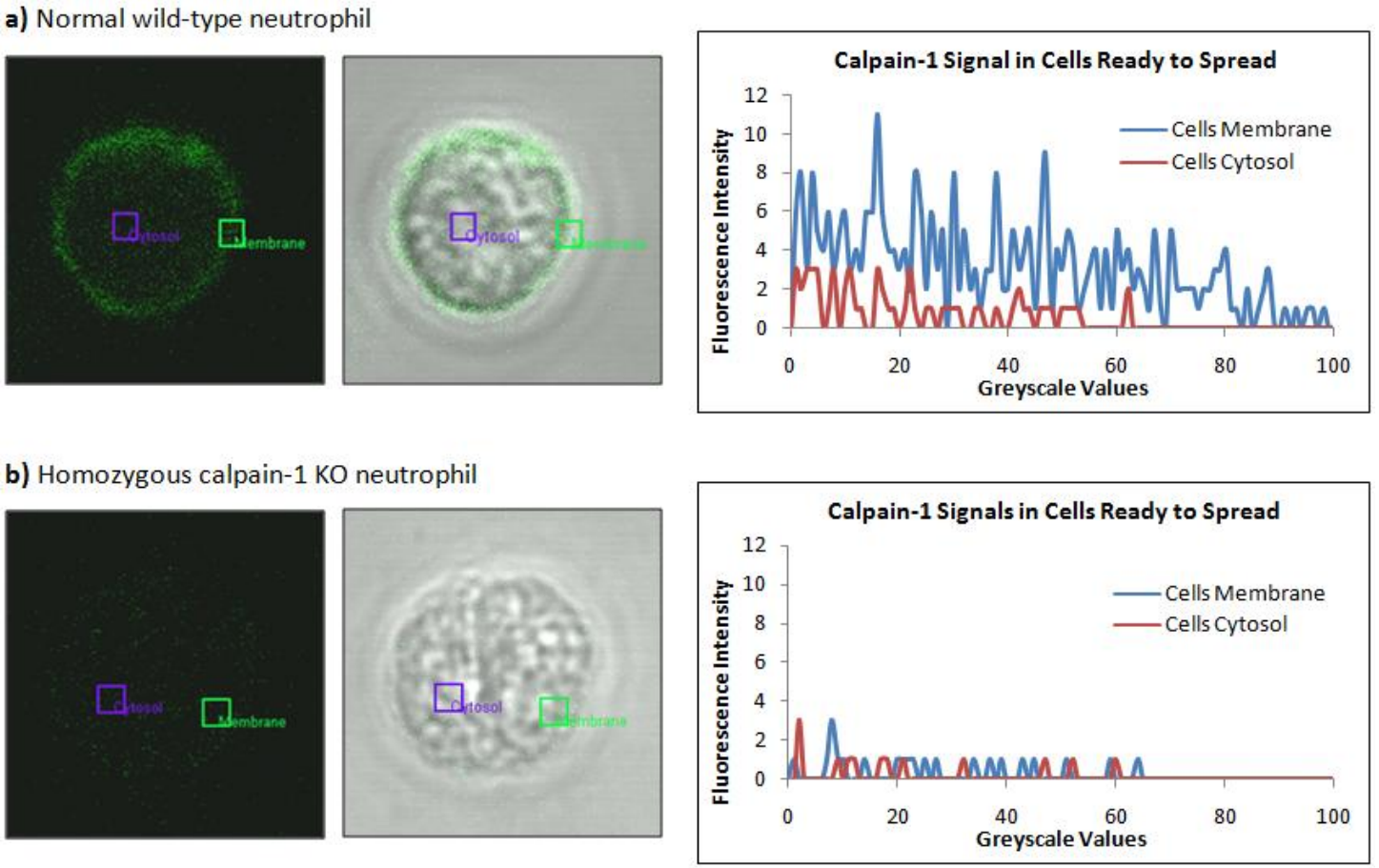


Figure 6.4.4.1: The picture and graph representing calpain-1 fluorescence signals. The picture and graph above represents the calpain-1 fluorescence signals on adhered (a) normal wild-type and (b) homozygous calpain-1 KO cells ready for spreading. Normal wild-type neutrophils showed the location of calpain-1 based on the fluorescence signals which were highly expressed on the membrane as compared to the signals in the cytosol. This effect is also compared to the homozygous calpain-1 KO neutrophil fluorescence staining which showed low fluorescence signals on both the plasma membrane and in the cytosol.

6.5. Discussion

The results gathered from the experiments in this chapter indicate that knocking-out calpain-1 altered neutrophil behaviour *in vitro*. In general, neutrophils isolated from either normal wild-type or homozygous calpain-1 KO mice still have the same fundamental abilities such as adhesion and spreading. The neutrophil populations of both normal wild-type and homozygous calpain-1 KO mice were shown to have similar size when they adhered onto the glass coverslips but not spreading. It was also concluded that neutrophil adherence and spreading in response to fMLP could function regardless of whether calpain-1 is present or not. However, the qualitative assessment on spreading area of individual neutrophils showed the evidence of cellular disruption in homozygous calpain-1 KO neutrophils. The results from the cell spreading work showed that calpain-1 was involved in the rate of neutrophil spreading. These results imply that the competency of homozygous calpain-1 KO neutrophils to spread on the glass coverslips failed to achieve their full capacity and have been disturbed. Taken together, the results indicate that neutrophil's response mechanisms via the fMLP receptor were still intact, but the homozygous calpain-1 KO cells did not have a comparable spreading capacity as the normal wild-type cells.

As it has been reported previously, releasing the intracellular Ca^{2+} store in human neutrophils through IP_3 uncaging subsequently prompted the cells to signal Ca^{2+} and spread (Pettit and Hallett 1998; Hillson et al. 2006). This approach provides the opportunity to study the cell's capability to spread through mechanisms which are different from the fMLP stimulation pathways. Uncaging IP_3 in the murine neutrophil has proven that the release of intracellular Ca^{2+} storage in the homozygous calpain-1 KO netrophils is similar to the Ca^{2+}

signalling recorded in the normal wild-type cells. The live cell recordings of the cells with a single cytosolic Ca^{2+} peak suggested that signalling via IP_3 pathway was only sufficient to cause the cells to slightly expand but not enough to induce full spreading in either normal wild-type or homozygous calpain-1 KO cells. In a highly evolved physiological system such as human, the degree of this difference in cellular reactions towards the uncaged IP_3 suggests the inter-species variation that exists between human and murine neutrophils.

The multiple-peaks of Ca^{2+} signals in both of the cell samples implies that there are other signalling mechanisms which are associated with the multiple rise of Ca^{2+} level before the mouse cells could achieved proper spreading. Both normal wild-type and homozygous calpain-1 KO neutrophils reacted in the same manner with either a single or multiple peaks of Ca^{2+} levels. This finding suggests that the increase of cytosolic Ca^{2+} levels by releasing the intracellular Ca^{2+} storage through the IP_3 signalling pathway is only sufficient to promote the cells to expand slightly, but when this effect is coupled with another intracellular signalling mechanism, it will cause multiple Ca^{2+} influx into the cells (demonstrated by multi-peaks of Ca^{2+} signals) and eventually promotes the cells to flatten and spread. This means that the cell's reactions and abilities to spread via the multiple Ca^{2+} peaks signalling pathway were similar even in the absence of calpain-1. The results signify that the cells responses towards the fMLP stimulus or IP_3 uncaging had the same effects on their behaviour. Either after the fMLP addition or after IP_3 uncaging with multi-peaks of Ca^{2+} signals, the calpain-1 absence did not hinder the cells spreading machinery itself. These findings imply that once the cell is stimulated by fMLP or the Ca^{2+} influx is induced through the IP_3 uncaging, calpain-1 becomes active and begins to cleave their substrates which links the actin filaments and the plasma membrane. This would then liberate the cell's membrane which allows them to expand and

spread. With the smaller surface areas of spreading generated by homozygous calpain-1 KO neutrophils, it means that the absence of calpain-1 has compromised the cells competency to spread fully. This could contribute towards the observed defect in the trans-endothelial migration shown in Chapter 4.

The location of calpain-1 which was predominantly on the edge of adhered neutrophils (on the plasma membrane) implicated the role of calpain-1 during the cell spreading process. Furthermore, failure to detect the presence of calpain-1 in homozygous calpain-1 KO neutrophils indicates that the enzyme was not involved in the spreading process. The ability of homozygous calpain-1 KO neutrophils to spread means that the cells may have followed different/alternative mechanisms involving different enzymes or intracellular signalling pathways. As the results show, the alternative mechanisms of action or signalling pathways to stimulate cell spreading are less effective when compared to the effects shown by the normal wild-type neutrophils. In conclusion, knocking-out calpain-1 has disrupted the ability of neutrophils to achieve their full spreading capacity and hampered the ability to migrate across the endothelial cells as well. The defect in neutrophil's ability to change shape and spread fully could also interfere with their ability to migrate in a directed manner towards the chemoattractant gradient in a process known as chemotaxis.

Chapter 7

Neutrophil Chemotaxis and the Effect of Calpain-1 Deletion

7.1. Introduction

Since the defective trans-endothelial migration showed by neutrophils deficient in calpain-1 (see Chapter 4) involves adhesion, shape change and chemotaxis, a defect in the cell's directional migration process or also known as chemotaxis could be a contributory factor. Thus, it was important to establish whether the ability of neutrophils from calpain-1 null mice have an altered ability to undergo chemotaxis.

7.1.1. Neutrophil Chemotaxis

Cell movement and progression is one of the essential characteristics of neutrophils which normally follow the chemoattractant signals generated in the body. Once signals are produced by the inflamed tissues, they will trigger the circulating blood neutrophils to transmigrate and then move towards the site of infection or inflammation in a chemotaxis motion. This chemotaxis progression will direct the cells to the site of action before they execute their anti-bacterial tasks. Neutrophils progress in a directional movement which is also referred to as chemotactic movement following the introduction of a chemoattractant such as IL-8 (Hammond et al., 1995) or fMLP. However, there are conflicting views as to whether calpain activation occurs during chemotaxis and whether it has a positive or negative effect.

Neutrophil chemotaxis and directional persistence towards fMLP and IL-8 gradients were decreased after inhibiting calpain, and at the same time promote rapid chemokinesis (Franco and Huttenlocher, 2005; Lokuta et al., 2003). Calpain was suggested to have a

negative regulatory function in resting neutrophils and inhibiting this enzyme subsequently facilitated membrane projections permitting random migration. Katsube et al. (2008) also suggested that the calpain inhibitor activated neutrophil chemotaxis. They reported that creating the concentration gradients of the calpain inhibitor resulted in neutrophil migration towards the tips of the micropipettes. These authors suggested that the cells have reacted in a chemotactic manner. This is contrary to the effect found in other types of cells where calpain inhibition essentially inhibited cell migration (Glading et al., 2002).

In view of the fact that calpain-1 has been found to be the predominantly active enzyme of the two key calpains in neutrophils, the generation of the homozygous calpain-1 KO mice gives the opportunity to investigate the effects of calpain-1 absence on neutrophils during chemotaxis and to examine whether variation does exist in comparison to normal wild-type neutrophils.

7.2. Aims of the Chapter

The aims of the work described in this chapter were to:

1. Record the movements of isolated neutrophils from normal wild-type and homozygous calpain-1 KO mice following the chemoattractant gradients.
2. Quantitatively analyse both normal wild-type and homozygous calpain-1 KO neutrophil progression by tracking the cell's movement following the gradient concentrations.
3. Establish the chemotaxis or chemokinesis reaction of normal wild-type and homozygous calpain-1 KO neutrophils following the chemoattractant gradient.

7.3. Methods

This methodology section describes the protocol and techniques used to perform the experimental work in this chapter. The methods include; tracking neutrophil progression towards an the fMLP gradient in a chemotaxis assay and analysing the direction of migration of the normal wild-type and homozygous calpain-1 KO neutrophil.

7.3.1. Chemotaxis Assay Using the Micropipette Injection Method

Chemotaxis was recorded using the Leica LCS inverted confocal microscope to image cells migrating towards a chemoattractant in the micropipette. Prior to this experiment, neutrophils isolated from normal wild-type and homozygous calpain-1 KO mice were suspended in HBK medium. To create the chemoattractant gradient, fMLP (2µM in HBK) was filtered with the syringe filter (0.5µm pore size) before placing it in micropipettes. The fMLP solution was also mixed with a fluorescence dye, lucifer yellow to allow visualisation and measurement of the concentration gradient, as well as ensuring the micropipettes are not blocked during the assay. At the beginning of the assay, neutrophils were placed onto glass coverslips on a heating stage and left for about 5 to 10 minutes to allow the cells to adhere. fMLP was then loaded into the micropipette and tightly secured to the Eppendorf Femtojet (Eppendorf, Germany) pressure controller for microinjection before carefully adjusting it to the field of vision (40X magnification) of the cell population. Once the set-up is ready, fMLP was slowly injected (15hPa (hectopascal) pressure) into the extracellular medium to create the concentration gradient. The cell movements were recorded for 10 minutes (500 frames) and subsequently analysed.

7.3.2. Cell Tracking and Chemotaxis Analysis

The videos of neutrophil movement were analysed using software "plugin"for Image J which allowed manual cell tracking. Analysis by Image J is based on the coordinates of the cells at the beginning and at the end of the experiment on an X and Y axis plot. Before all the cells were individually tracked, the coordinates for the micropipette tips were plotted and identified as the central point for chemoattractant gradients which also acts as indicator of the chemotaxis capability. 100 cells from the normal wild-type and homozygous calpain-1 KO mice were tracked using Image J, and the data were analysed with the Ibidi® chemotaxis and migration tool software. The data were plotted on graphs (X and Y axis in µm/second) with all the cells starting coordinate set at (0,0). The cell chemotaxis capability was determined by calculating the migration Directness for the cell populations, identifying their Centre of Mass, and evaluating their Forward Migration Index (FMI). The results from both normal wild-type and homozygous calpain-1 KO cells were then compared for differences. The data are reported as means from five different experiments and were compared by one-way analysis of variance. The comparisons were made using Student's *t*-test for paired values and the statistical significance set at $p<0.05$.

7.4. Results

7.4.1. Neutrophil Migration and Chemotaxis

The progression of neutrophils in motion was individually tracked from their starting position and followed through until the end of the recorded experiments regardless of their direction. As a general rule for this work, murine neutrophil chemotaxis was characterized by movement of the cells from the left side of the microscope field of view towards the tips of the micropipette (containing fMLP) on the right side. The concentration gradients of fMLP were visible with the fluorescence marker of the Lucifer yellow and measured accordingly (Figure 7.4.1.1). The movement of neutrophils in the direction of the region with high fMLP concentration indicated chemotactic locomotion. As observed, both of the neutrophils samples showed that they were actively responsive to the fMLP gradient. The tracked neutrophils from normal wild-type (Figure 7.4.1.1) and homozygous calpain-1 KO (Figure 7.4.1.2) mice proved to be capable of migrating towards the concentration gradient created, although some cells also moved away from the micropipette tips. A number of cells from both normal wild-type and homozygous calpain-1 KO animal were firmly adhered on the surface of glass coverslips with some cells showing random membrane projections and movements which resembles the chemokinesis reaction.

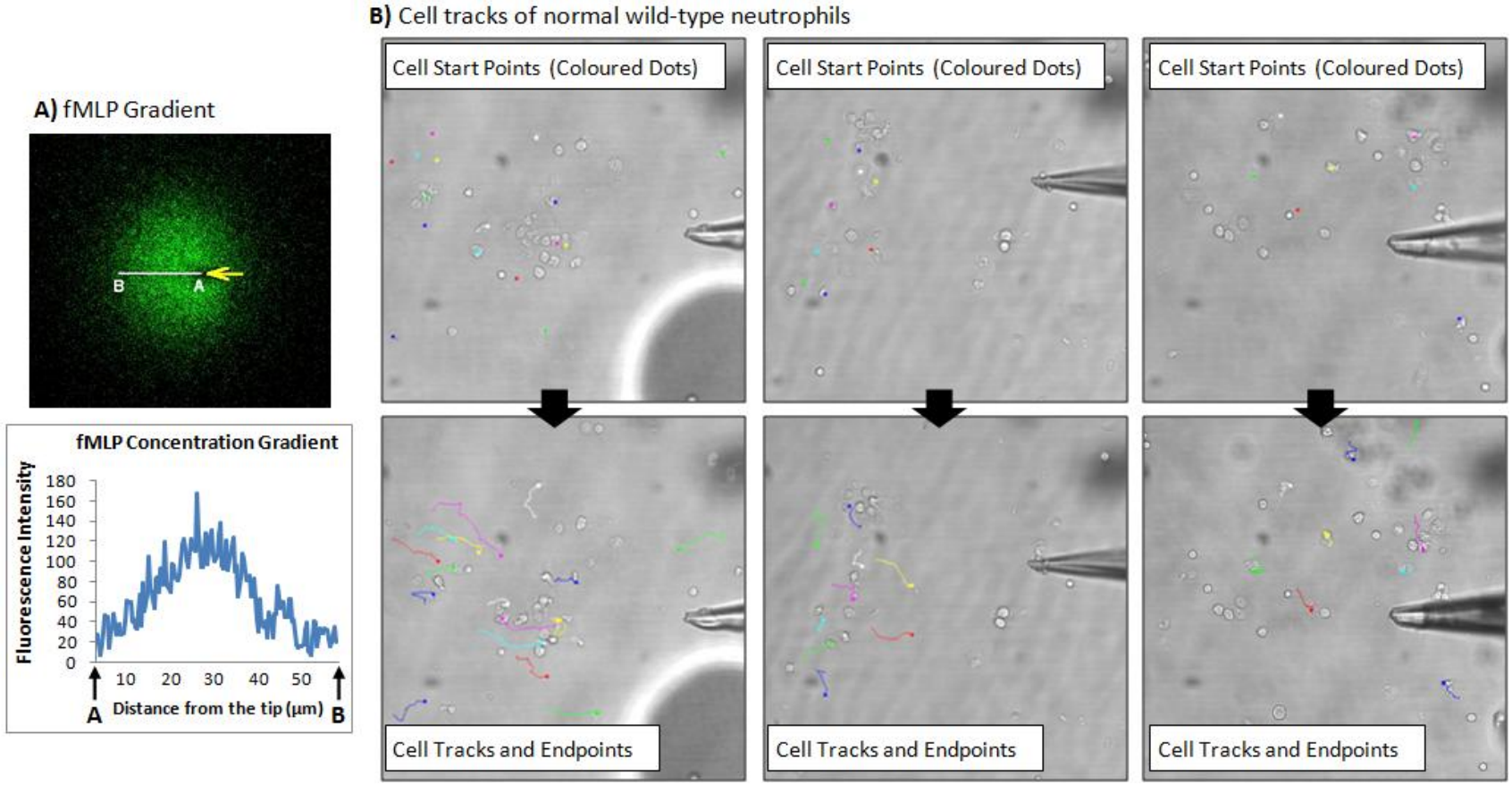


Figure 7.4.1.1: The fluorescence image of the fMLP gradient after the injection. Figure in (A) is the fluorescence image of the fMLP gradient shortly after the injection. The fluorescence intensity is determined along the line from point A to point B and plotted on the graph to illustrate the concentration gradients of the probe. Pictures in (B) represent normal wild-type neutrophils before and after the fMLP microinjection. Cells in motion were identified before being individually tracked from their starting spot and their trails were traced (as shown by the coloured tracker) until the end of the recording. It shows that the majority of the cells are progressing towards the tips of the micropipette with some cells moving in a non-directed manner or showing chemokinesis reaction.

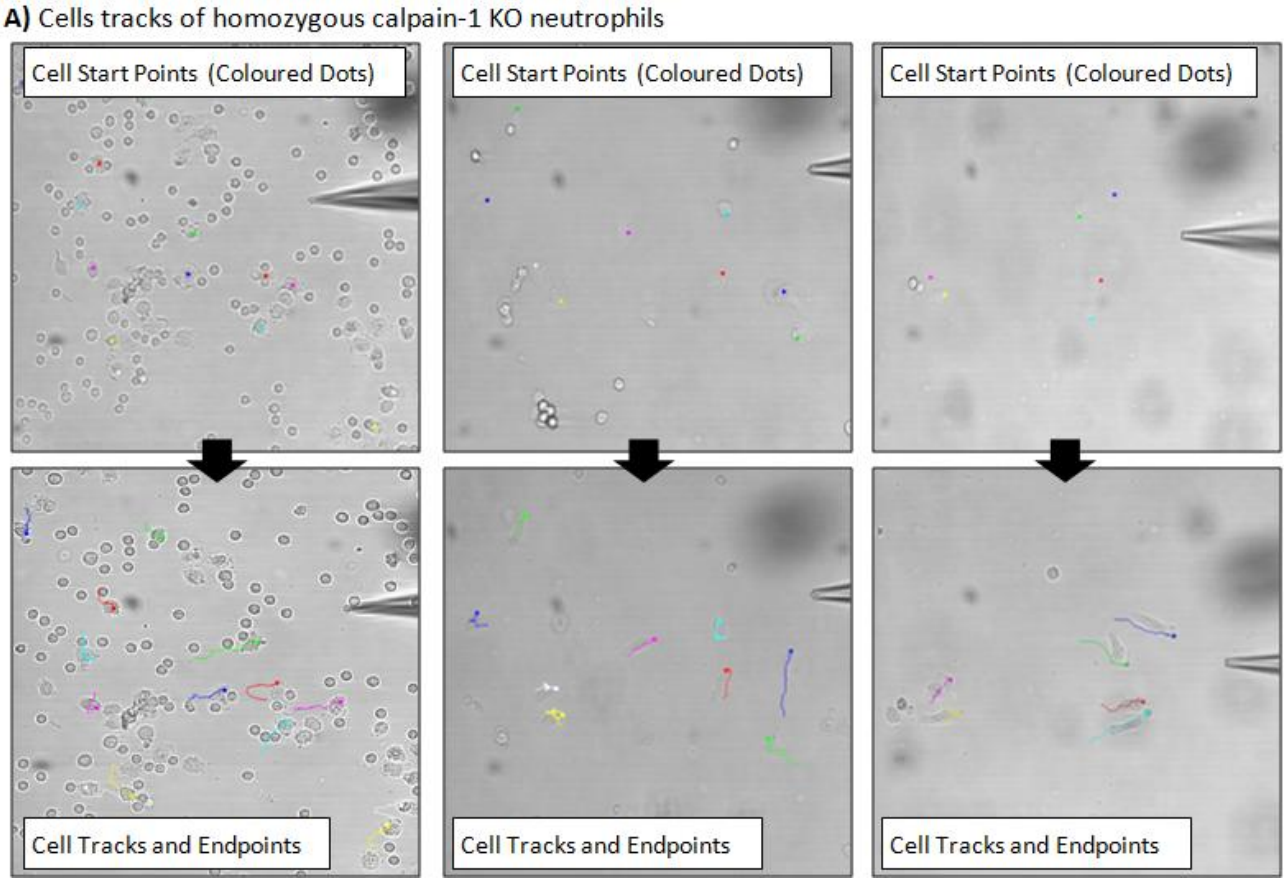


Figure 7.4.1.2: The pictures of homozygous calpain-1 KO cells before and after fMLP. The pictures above portray the homozygous calpain-1 KO cells (with other white blood cells) before and after the introduction of fMLP. Migrating neutrophils were identified and individually tracked from their starting spot (coloured tracker) as with the normal wild-type cells. These homozygous calpain-1 KO cells showed similar progression patterns as normal wild-type neutrophils by moving towards the micropipette tips (fMLP gradient) with some neutrophils showing non-directed movement or chemokinesis reaction. Thus, it means their "sense of direction" and their capability to undergo chemotaxis is still intact despite the absence of calpain-1 enzyme.

7.4.2. Distances, Directness, Centre of Mass and Forward Migration Index (FMI)

The quantitative analyses were performed by setting the bar scales (in µm) for the images according to the Leica confocal microscope setting for accurate measurement. The trajectory plots of the moving cells on the X and Y axis, were assessed for their accumulated distance which measured total distance of the cell trails and the measurement of Euclidean distance which calculated the straight line between the cell's starts and ends points (Figure 7.4.2.1). The results showed that maximum accumulated distance travelled by normal wild-type and homozygous calpain-1 KO cells were 32.08µm and 25.36µm respectively, and the average accumulated distance of homozygous calpain-1 KO cells measured at 12.55µm (+/- 0.44 S.E.M) was slightly higher than 12.22µm (+/- 0.62 S.E.M) for the normal wild-type cells. There was no significant difference between them (Figure 7.4.2.6).

The directness value is calculated by comparing the Euclidean distance of each cell to their accumulated distance. This number represents a measure of the directness of the cell trajectories during migration, although this is not a direct parameter for judging chemotaxis (Semmling et al., 2010). However, it can be used to characterize straightness of migration, which is related to chemotaxis and chemokinesis. The cell's directness value that is close to 1 indicates straight migration of neutrophils towards the micropipette tips and the injected fMLP (Figure 7.4.2.2). The directness values of normal wild-type and homozygous calpain-1 KO neutrophils were 0.67 and 0.61 respectively (Figure 7.4.2.6). This demonstrates that the migration trajectory of either cell group has no difference.

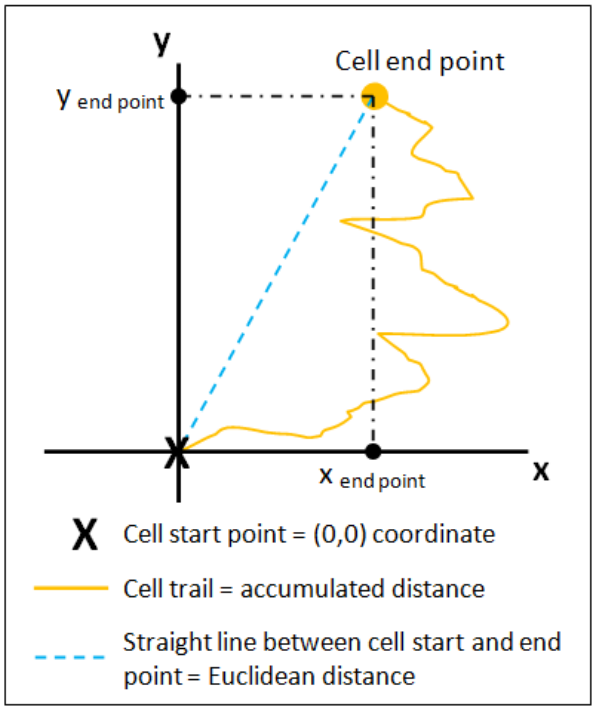


Figure 7.4.2.1: The definition of accumulated distance and Euclidean distance. This figure explains the definition of accumulated distance and Euclidean distance of locomoting cells from the starting point until the end of their movement on an X and Y axis.

Calculation for Cell Directness =

$$\frac{\text{Distance}_{\text{Euclidean}}}{\text{Distance}_{\text{Accumulated}}}$$

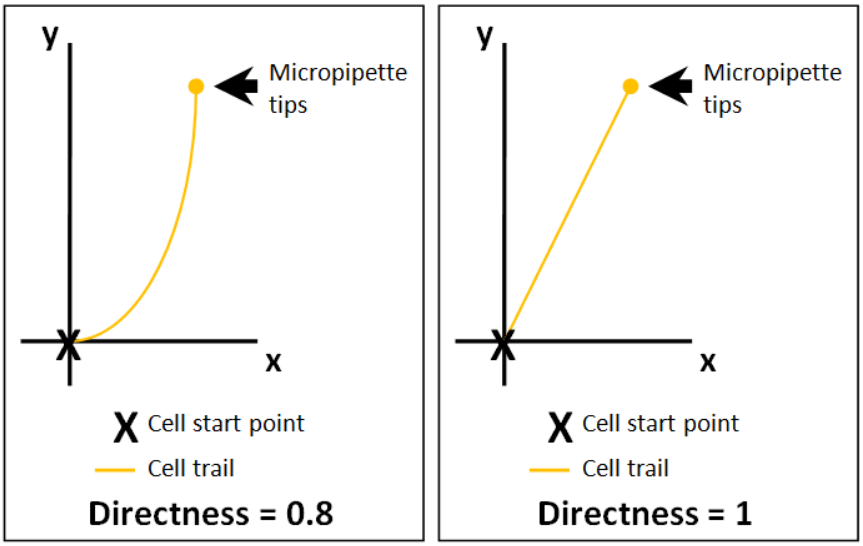


Figure 7.4.2.2: Formula of directness based on Euclidean and accumulated distance. This formula explains how directness is calculated based on the Euclidean and accumulated distance measurements. The graphs are examples of the directness (D) value of the cells migration with a curvy and straight trail (cell end points in both graphs are at the same coordinate).

The centre of mass for the cell population was determined by plotting the migration trails from the starting coordinate of (0,0) to the endpoints. This centre of mass represents only one point, which is the averaged point of all the cell's endpoints. The value for centre of mass is a strong parameter for evaluating chemotaxis. The differences in this centre of mass value at the start of the experiment and at the end is known as the displacement (or length) of the centre of mass (Zengel et al., 2011). This value represents the migrations length for all neutrophils observed in this experiment. The data analysis showed comparable number of neutrophil populations of normal wild-type (69 cells) and homozygous calpain-1 KO (66 cells) have migrated in the same direction (from left to right of the microscope fields) towards the fMLP concentration gradient (Figure 7.4.2.3). The displacement value for the normal wild-type cell populations at 3.53µm was higher than the homozygous calpain-1 KO cells at 3.02µm, and showed a significant difference (p<0.05, one way ANOVA Student's t=test; n = 5) between the two parameters (Figure 7.4.2.6). This suggested a "stronger" chemotaxis response in the normal wild-type neutrophils and in some measure signifies interruption to the calpain-1 knock-out cell's ability to undergo chemotaxis.

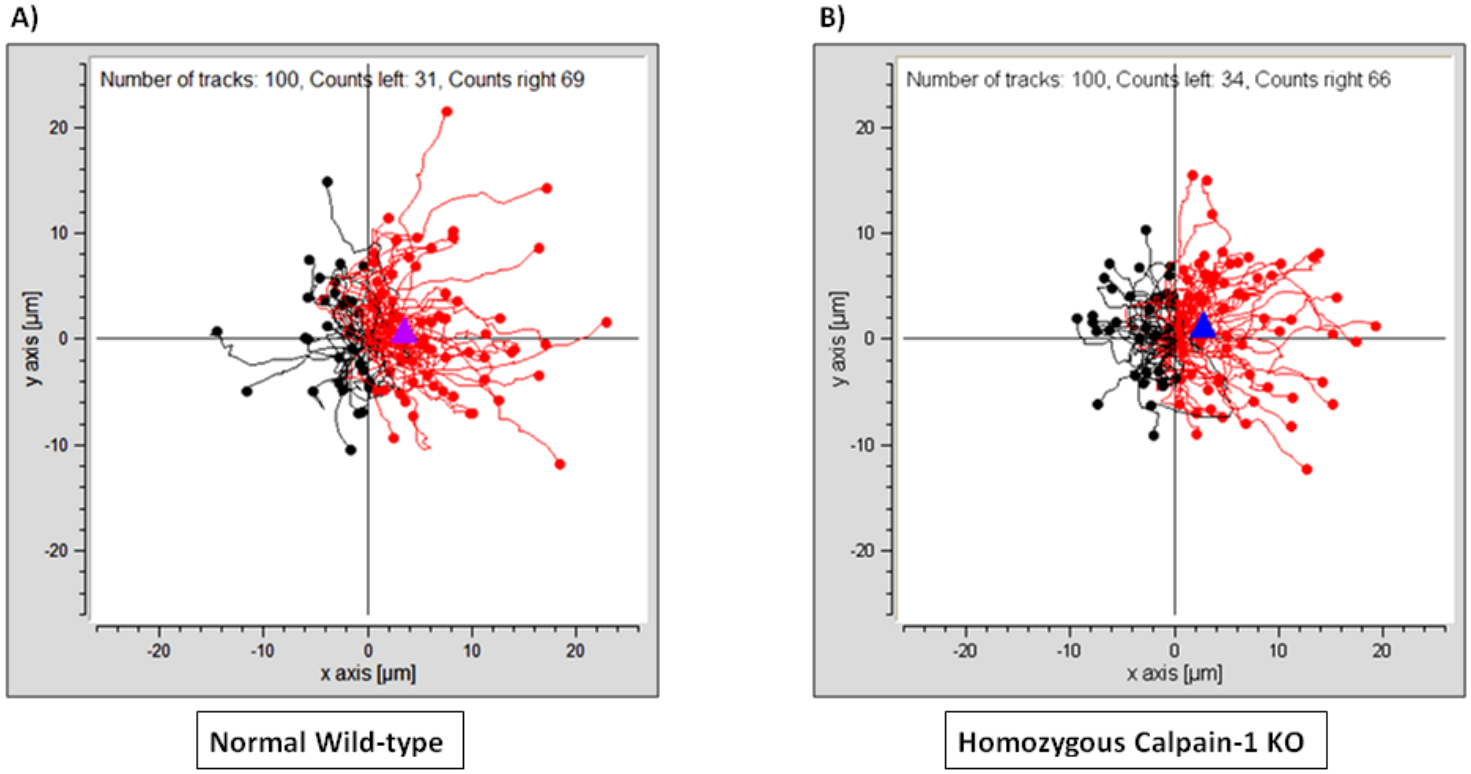


Figure 7.4.2.3: Migration plots and the centre of mass of the chemotaxis assay. The migration plots above (A and B) illustrate the centre of mass (marked with the blue triangles) for both normal wild-type and homozygous calpain-1 KO cell populations at the end of the chemotaxis experiments. As the plots shows, the majority of the cells have migrated to the right side of the grids (69 normal wild-type cells and 66 homozygous calpain-1 KO cells) towards the micropipette tips from their starting coordinates of (0,0). This indicates that both sets of cells have completed the same chemotaxis migration with the presence of fMLP gradient.

The Forward Migration Index (FMI) represents the efficiencies of neutrophil forward migration up the chemoattractant gradient, and how it relates to the direction on both X and Y axis (Foxman et al., 1999). Depending on the direction the cell population has travelled, the FMI values on both axes can be either positive or negative. The FMI values are only relevant when chemotaxis is parallel to, or perpendicular to the X and Y axis relative to fMLP gradient (Figure 7.4.2.4). In this experiment, the neutrophil populations were expected to drift to the right of the fields, therefore, the FMI parallel value is measured on the X axis (FMI_X) and the FMI perpendicular value is measured on the Y axis (FMI_Y). Strong chemotaxis is characterized by high FMI_X (either positive or negative value) and FMI_Y that is close to zero. The FMI values for the normal wild-type cells was recorded as FMI_X = 0.2415 and FMI_Y = 0.0869, whereas, FMI values for homozygous calpain-1 KO cells were FMI_X = 0.1559 and FMI_Y = 0.1136. This result means that the normal wild-type cells populations have higher FMI_X value of 0.2415 than homozygous calpain-1 KO cells of 0.1559. Furthermore, the FMI_Y value for normal wild-type neutrophils (0.0869) is much closer to zero than the homozygous calpain-1 KO neutrophils (0.1136). The figures show that the normal wild-type neutrophils have a higher directional movement towards the fMLP gradient than homozygous calpain-1 KO neutrophils, but this effect was not significantly different (Figure 7.4.2.6).

The Rayleigh test is a common statistical test is used to analyze the uniformity of a circular distribution of points, or in this case the cell endpoints. This Rayleigh test for vectors data includes the distances of the points from the origin (Zengel et al. 2011). The Rayleigh test was designed to test the null hypothesis: namely, uniformity with respect to the equal circular distribution of cell endpoints. The null hypothesis is rejected if p-value smaller than p=0.05. This test strongly depends on the numbers of cells which are being analyzed. The p-

value of Rayleigh test for both normal wild-type and homozygous calpain-1 KO neutrophils were summed at 6.52×10^{-6} and 1.45×10^{-4} respectively, indicating that both of the neutrophil populations have a significant non-uniform distribution (Figure 7.4.2.5).

Neutrophils from both normal wild-type and homozygous calpain-1 KO samples were further analysed based on the number of migrated cell endpoints which were separated by the Y axis (total number of cells on the left and right side of the migration plots) using the chi-square test. The results showed a p-value of 0.651 ($p>0.05$), which means that there are no significant chemotaxis differences between both of the normal wild-type or homozygous calpain-1 KO cell populations. The velocity of normal wild-type and homozygous calpain-1 KO neutrophils that have migrated furthest from their starting points were recorded at 3.3μm/min and 2.64μm/min (at 37°C) respectively. This result showed a great difference in comparison to the speed of human neutrophil migration which was recorded at 24μm/min (at 37°C) (Hallett et al. 1989). This suggests the inter-species variation in chemotaxis ability between human and murine neutrophils.

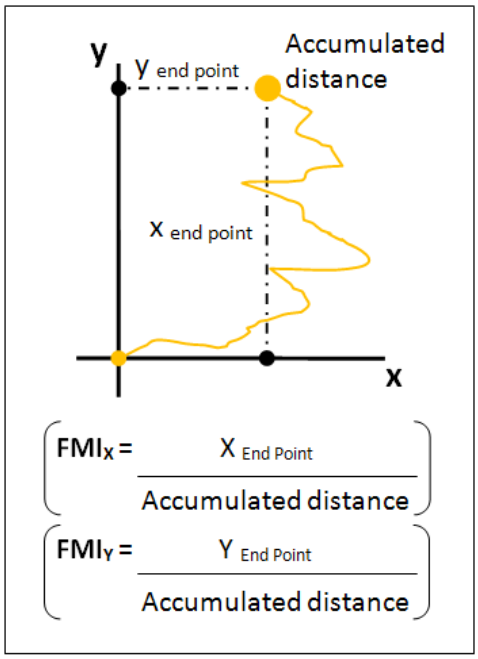


Figure 7.4.2.4: Mathematical formula for FMI calculations of the FMI_x and FMI_y value of migrating neutrophils. This illustration explains how the FMI_x and FMI_y values of migrating neutrophils is calculated.

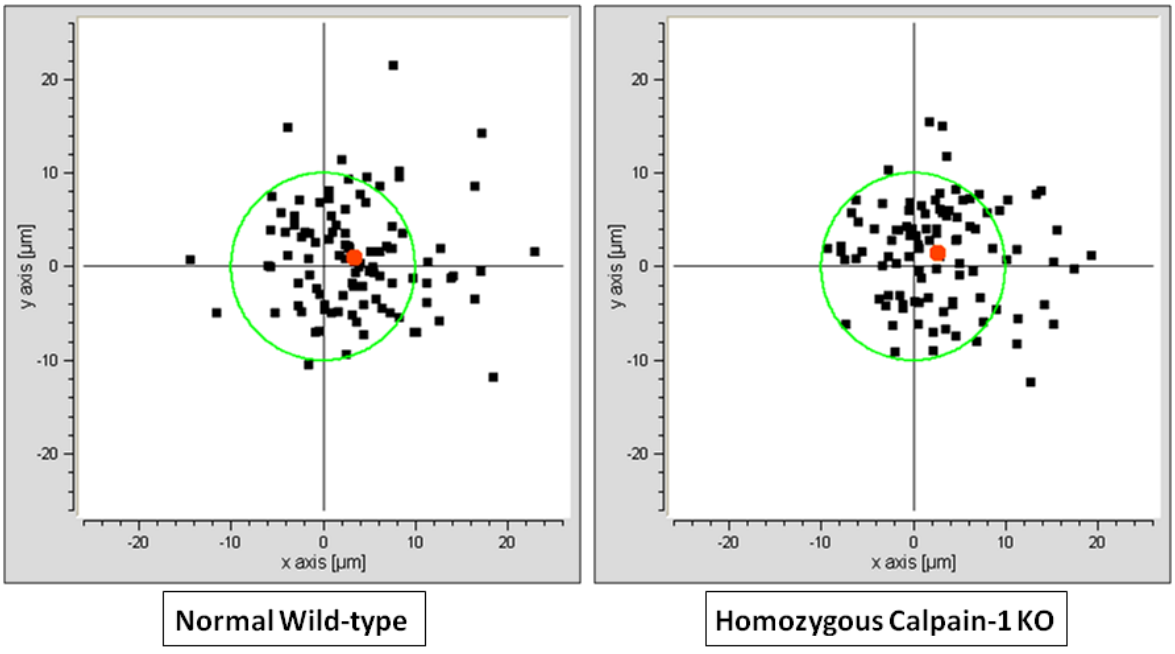


Figure 7.4.2.5: The plot of cell endpoints distribution with the Centre of Mass. This figure shows the cell endpoints distribution of normal wild-type and homozygous calpain-1 KO neutrophil populations, with the Centre of Mass marked by the red dots. The circular fields (drawn based on the 0,0 axis) on both of these migration plots illustrated that the cell distributions are more on the right side of the grid where the chemoattractant gradient was created.

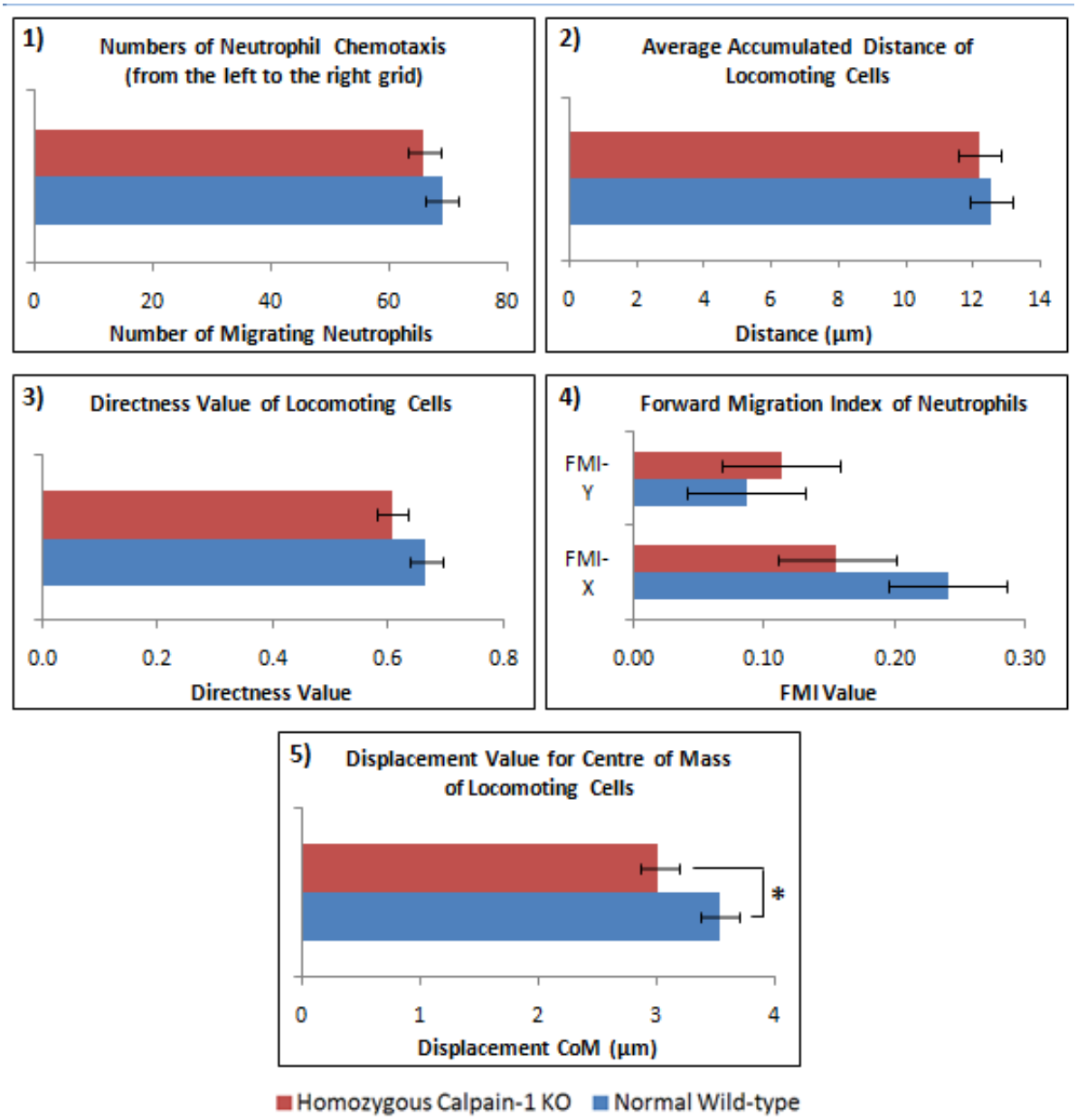


Figure 7.4.2.6: The graphs summarizing the results from the chemotaxis experiments. In general, it shows here that calpain-1 deficient neutrophils can still undergo chemotaxis when chemoattractant was introduced. The number of cells that underwent chemotaxis (Graph 1) and the average accumulated distance of migration (Graph 2) have no significant differences. This means that both normal wild-type and homozygous calpain-1 KO cells have equal ability to migrate in a chemotactic manner. This is supported by the directness value (Graph 3) and the Forward Migration Index (Graph 4) of the cells which did not show any differences. However, displacement value for the Centre of Mass during migration (Graph 5) showed significantly different results between normal wild-type and homozygous calpain-1 KO neutrophils. This indicates that although the displacement values between the two cell samples showed a discrepancy, the ability of neutrophils to undergo chemotaxis was not disrupted by the absence of calpain-1. These results are expressed as means and are representative of five separate experiments. *p<0.05 compared to the normal wild-type mice (analysis of variance using Student's t-test).

7.5. Discussion

The works presented in this chapter implied that the absence of calpain-1 did little to disrupt neutrophil chemotaxis. The live cell imaging of neutrophil migration towards fMLP gradients provided strong proof about the ability of homozygous calpain-1 KO neutrophils to perform chemotaxis. Although on average the directness value of the homozygous calpain-1 KO suggested that their "sense of direction" is still intact, the population shifts from the cell starting points to their endpoints (towards the chemoattractant gradient) suggested a flaw in the chemotaxis process. This effect could be caused by the cell's shortcomings with their "Velcro" attach-release ability of the cell membranes during migration. This could be due to the inefficiency of calpain cleavage actions on the anchor between their substrates and the integrin molecules.

The integrin molecules in resting leukocytes are constitutively bound to the cell's actin cytoskeleton via protein linker (Sampath et al., 1998). It has been proposed that neutrophil motility involves the internalization of integrin into endocytic vesicles from the rear of the cell and recycled forward (Lawson and Maxfield, 1995). This indicates that the cells are free to travel once their membrane is released from the tether. It can be said that the release of the "Velcro" in homozygous calpain-1 KO cells was not as good as in normal wild-type cells due to the calpain-1 absence. However it shows that the normal wild-type and homozygous calpain-1 KO neutrophils can progress in a chemotactic manner. The lack of calpain-1 has also showed the inability for the cells to achieve their utmost spreading capacity (Chapter 6). The findings presented in this chapter did not demonstrate any major influence of calpain-1 in chemotaxis. By taking this result together with the outcomes from

previous chapters, it implies that calpain-1 influences neutrophil's ability to change shape but not their chemotaxis process. This suggests that there could be a different mechanism of action for the cells to regulate their directional senses, and this depends on the release of cell membrane. Calpain cleavage on the focal adhesion complex which links the cellular actin cytoskeleton to the extracellular matrix, promotes the reorganization of the molecular components and consequently leads to the weakening of cellular adhesion and increased motility (Carragher and Frame, 2002).

It could also be said that regardless of which mechanism of action is involved in order to liberate the plasma membrane of either normal wild-type or homozygous calpain-1 KO neutrophils, these neutrophils are capable of undergoing chemotaxis once the adhesion complex is cleaved. In conclusion, the absence of calpain-1 did little to disrupt neutrophils chemotaxis ability and calpain-1 might not be directly involved with coordinating neutrophil chemotaxis. The outcomes gathered from the previous chapters leads to the question of the extent of calpain-1 role in perhaps one of the most important function in neutrophils, their phagocytosis ability.

Chapter 8

Phagocytosis and Calpain-1

8.1. Introduction

The effects of calpain-1 depletion on trans-endothelial migration, cell spreading and to a lesser extent chemotaxis, all point to a common central defect, namely the ability of neutrophils to change shape. This opens the question as to whether the phagocytosis process, which also involves changes in neutrophil shape, would also be affected. The work in this chapter therefore aims to investigate whether a role for calpain-1 could be found in phagocytosis by establishing the effect of calpain-1 deficiency on the phagocytosis process.

8.1.1. Neutrophil Phagocytosis

Neutrophils have been established as the first line of defence by the immune system against infecting microorganisms into the body. One of the most prominent characteristics of these cells is their ability to engulf foreign particles in a process called phagocytosis (Lee et al., 2003; Segal, 2005). Phagocytosis involves capture of targeted particles, internalisation and killing. In order for such foreign particles to be targeted by neutrophils, they are initially coated with opsonin such as IgG and C3b in a process called opsonisation (Frank and Fries, 1991). Foreign particles such as the opsonised zymosan, either in a close contact or in cell suspension are a useful means of studying phagocytosis as they are readily ingested by neutrophils (Roos et al., 1981). Without this opsonisation process, the recognition and killing of the invading microorganisms is inefficient.

Contact between opsonised microorganisms and neutrophils result in the projection of localised regions of neutrophil plasma membrane to form pseudopodia which then engulf

the entire microorganism. Cells observed under the SEM showed that adherent neutrophils incubated together with opsonised zymosan at 37°C appeared to make contact after just 15 seconds (MacRae and Pryzwansky 1984). After 30 seconds the zymosan particles have been partially engulfed into the open cavities formed by the neutrophil pseudopodia extensions. By 1 minute, the particle is ingested in an intracellular vacuole, also known as the phagosome, before losing their membrane projections and after 3 minutes showing bulging outlines of the internalized zymosan. It is believed that the formation of these membrane projections involves untethering the cell membrane "wrinkles" from the actin filaments by cleaving the molecules that hold the membrane together, and the enzyme calpain has been implicated in this process.

A rise in cytosolic free Ca^{2+} has been recorded in neutrophils during phagocytosis and relies mainly on the release of intracellular Ca^{2+} storage, however this is not a prerequisite for phagocytic activity (Theler et al., 1995). The Ca^{2+} signals are consistently observed when neutrophils are presented with opsonised particles. There is no change in Ca^{2+} concentration during the pseudopodia extensions phase, but a large Ca^{2+} change is detected in the cytosol before the cell rapidly encloses the particle and retracts its pseudopodia (Dewitt and Hallett, 2002). By taking into consideration that calpain is a Ca^{2+} activated enzyme, it is fundamental to understand their role in phagocytosis and how the rise in cytosolic Ca^{2+} concentration is associated with the pseudopodia extension. The successful generation of the homozygous calpain-1 KO mouse colony has provided the opportunity to investigate the role of calpain-1 in the phagocytosis process, and to distinguish any discrepancies as compared to the normal wild-type neutrophils.

8.2. Aims of the Chapter

The aims of the work described in this chapter are to:

1. Study the ability of neutrophils from the normal wild-type and homozygous calpain-1 KO mice to phagocytose when presented with opsonised zymosan.
2. Quantitatively measure the capability of normal wild-type and homozygous calpain-1 KO neutrophil populations to phagocytose the opsonised zymosan in cell suspensions.
3. Measure and analyse the Ca^{2+} signals during phagocytosis in both normal wild-type and homozygous calpain-1 KO neutrophils.
4. Detect and determine the cellular location of the calpain-1 enzyme in both normal wild-type and homozygous calpain-1 KO neutrophils during phagocytosis.

8.3. Methods

This methodology section describes the protocol and technique used to perform the experimental works for this chapter. These methods include; the neutrophil phagocytosis by presenting opsonised zymosan in suspension and by micropipette manipulation, measuring the Ca^{2+} signalling during phagocytosis, and detecting the expression of calpain-1 in normal wild-type and homozygous calpain-1 KO neutrophil undergoing phagocytosis.

8.3.1. Neutrophils Phagocytosis of Opsonised Zymosan in Suspension

Mouse serum for opsonising the zymosan particles was prepared by collecting blood through cardiac puncture from the normal wild-type mice and left to clot in the refrigerator

overnight in glass tubes. The supernatant from the clotted blood samples was transferred into small tubes and centrifuged at 350xG for 10 minutes to remove traces of RBC. The supernatant was separated from the pellet and further centrifuged at 350xG for 5 minutes. If necessary, this step was repeated until there are no more visible RBC residues present. The resultant supernatants were either used immediately or be kept in aliquots and stored in -20°C. At the start of experiments, zymosan particles from the stock (10mg/ml) were diluted with the previously prepared mouse serum in 1:9 ratios (mg/ml) and incubated at 37°C for 30 minutes to enable opsonisation to take place. The opsonised zymosan particles were then washed by repeated centrifugation to remove traces of serum, and resuspended in HBK medium at a concentration of 1mg/ml.

Isolated neutrophils from the normal wild-type and homozygous calpain-1 KO mice were prepared and suspended in HBK medium (1ml). The cells in suspension were added to the serum (C3bi) opsonised zymosan particles in 1:2 ratios (cell:zymosan particle) and then incubated at 37°C. The phagocytosis assay was stopped by removing the cell samples from the incubator and adding 4% (v/v) formaldehyde (in PBS) in a time course of 5 minutes, 15 minutes, 30 minutes, 1 hour, 1.5 hour, 2 hours and 3 hours. The cells were then centrifuged (150xG) onto the glass slides using a cytospin and stained with Hemacolor® Rapid Staining (Merck Chemicals Ltd.). Subsequently, the cells were observed under the microscope, and 200 cells were counted regardless of whether they had engulfed the particles (one or more zymosan) or not. Neutrophils which had undergone the phagocytosis process could be clearly distinguished by the presence of the zymosan particles engulfed inside the stained cell. The phagocytosis index (in percentage) is calculated based on the total number of cells counted (200) and the number of cells which have phagocytosed. The phagocytosis index

graphs for both normal wild-type and homozygous calpain-1 KO neutrophils were plotted and compared accordingly. The data are reported as mean from two different experiments and were compared by one-way analysis of variance. The comparisons were made using Student's *t*-test for unpaired values and the statistical significance set at $p<0.05$.

8.3.2. Phagocytosis of Opsonised Zymosan Using Micropipette Injector

Neutrophils from both normal wild-type and homozygous calpain-1 KO mice were isolated and loaded with Fura2 AM for Ca^{2+} signal detection (as described in Chapter 2). The samples were then loaded onto the glass coverslips and left for 5 minutes for the cells to adhere. Once the cells had properly adhered, opsonised zymosan particles were added to the cells on the glass coverslip. Under the confocal microscope, cells which were near zymosan were identified, and contact between neutrophil and the zymosan particles were induced using the micropipette and manipulator. Movies of the phagocytosis process were recorded and the graphs for Ca^{2+} signals were documented. Changes in the cell shape, particularly the phagocytic 'cup' formation and the increase of Ca^{2+} level were analysed using Leica LCS Lite software and Microsoft Office Excel 2007. The data are reported as mean of ten different neutrophils and were compared by one-way analysis of variance. The comparisons were made using Student's *t*-test for unpaired values and set at $p<0.05$.

8.3.3. Calpain-1 Fluorescence Staining on Neutrophils During Phagocytosis

This immunofluorescent calpain-1 staining work was prepared using a commercially available calpain-1 mouse monoclonal primary antibody (Santa Cruz Biotechnology) and the

goat anti-mouse IgG conjugated to fluorescein isothiocyanate (FITC) as secondary antibody. At the start of the experiment, isolated neutrophils were suspended in the HBK medium and added to the opsonised zymosan particles in 1:2 ratios (cell:zymosan particle), and then incubated for 10 minutes at 37°C. After that, the cells were removed from the incubator and left on the glass coverslips to adhere for about 15 minutes. Unbound cells were removed by washing twice with HBK medium. Cells were then fixed by adding 4% (v/v) formaldehyde (100µl) and left for 10 minutes at room temperature. The fixative solution was removed by washing the cells three times with (PBS). By adding 0.1% (v/v) Triton X-100 solution in PBS, the cells were permeabilized for 4 minutes at room temperature. The Triton solutions were subsequently removed by washing the cells twice with PBS.

To block non-specific binding of the antibodies, 100µl of 4% (v/v) horse serum diluted in PBS was added to the cells and left for 1 hour at room temperature. The primary antibody (calpain-1) was prepared by diluting in PBS with 4% (v/v) horse serum in a 1:100 µl ratio and added to the cells and left overnight at 4°C. The primary antibody was then washed three times with PBS (5 minutes for each wash). Secondary antibody (in 1:100 µl antibody to PBS-4% (v/v) horse serum ratio) was added and left in the dark for 1 hour at room temperature. Finally, the cell samples were washed twice with PBS (5 minutes each wash) to remove any unbound secondary antibody. Using the confocal microscope, immunofluorescence labelled neutrophils of the normal wild-type and homozygous calpain-1 KO samples were observed and recorded for calpain-1 presence.

8.4. Results

8.4.1. Phagocytosis Counts on Neutrophils in Suspension

Neutrophil populations of the normal wild-type and homozygous calpain-1 KO mice in suspension with the opsonised zymosan particles were shown to have a comparable phagocytosis capacity. Images of neutrophils laden with zymosan particles were taken and counted after the incubation time interval of between 5 minutes to 3 hours. The phagocytosis index percentage, which is based on the numbers of neutrophils that have completed phagocytosis of either one or more particles calculated against the total number of neutrophils observed (200 cells), showed similar graphical trends (Figure 8.4.1.1). After 5 minutes of incubation, the average percentage for normal wild-type neutrophil populations was recorded at 8% as compared to the 6% calculated with homozygous calpain-1 KO cells. In both of the samples, the numbers of neutrophils that completed phagocytosis increased at an approximately constant rate at each of the incubation time intervals.

After 30 minutes of suspension with the zymosan particles, the average percentage of the phagocytosis index reached 40% and 38% for the normal wild-type and homozygous calpain-1 KO cells respectively. After an hour of incubation, average phagocytosis index for normal wild-type neutrophils was 42% and for homozygous calpain-1 KO neutrophils it was 40%. From this period onwards, the average phagocytosis index percentage of neutrophils loaded with the zymosan particles continued to show a steady increase until the end of the experiment. Comparable average percentages of 80% and 82% phagocytosis index were recorded after the 3 hours incubation period for both normal wild-type and homozygous calpain-1 KO neutrophil populations respectively.

The outcomes means that the capacity for the homozygous calpain-1 KO neutrophil populations to complete the phagocytosis process is unchanged compared to the normal wild-type neutrophils despite missing the calpain-1 enzyme. The homozygous calpain-1 KO cell population also showed their efficiency in completing the phagocytosis process judging by the number of zymosan particles that they have engulfed, which did not exhibit much difference when compared to the normal wild-type cell counts (Figure 8.4.1.1). This finding gives an indication of the phagocytosis ability of normal wild-type neutrophils as compared to the homozygous calpain-1 KO cells. However, by examining the single cell reactions of the normal wild-type and homozygous calpain-1 KO neutrophil populations presented with zymosan particles, greater detail of the cell responses can be studied and analysed.

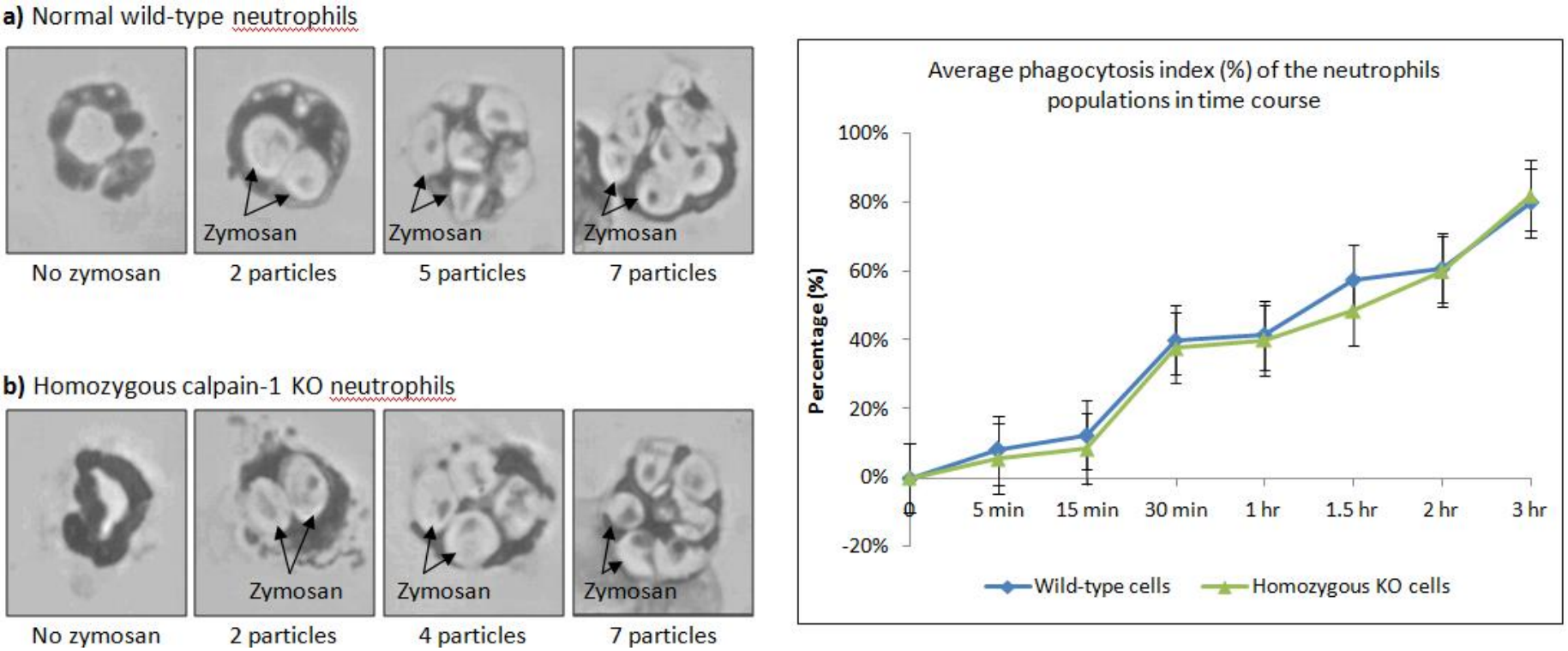


Figure 8.4.1.1: The images of non-phagocytose and phagocytose neutrophils. The images in (a) and (b) represents neutrophils of normal wild-type and homozygous calpain-1 KO mice respectively. These images depict non-phagocytose neutrophils without any zymosan particles in them, and images of neutrophils that have engulfed the zymosan particles. The graph represents average percentages of phagocytosis index of both normal wild-type and homozygous calpain-1 KO neutrophil populations suspended with zymosan particles during the incubation time course from 5 minutes to 3 hours. The data are reported as means from two different experiments and were compared by one-way analysis of variance. The comparisons were made using Student's *t*-test for unpaired values and the statistical significance set at $p<0.05$.

8.4.2. Phagocytosis of Opsonised Zymosan Using Micropipette Manipulation

Neutrophils isolated from normal wild-type and homozygous calpain-1 KO mice were able to complete their phagocytosis function when presented with the opsonised zymosan particles by micro-manipulation. However, there was a suggestion of a defect as one of the cells from homozygous calpain-1 KO mice failed to engulf the zymosan particles despite initially showing similar reactions as the other cells. The contact between the cells and the zymosan particles demonstrated that Ca^{2+} signalling mechanisms in homozygous calpain-1 KO cells is still intact, and showed the same effect as compared to the normal wild-type cells. This Ca^{2+} signals appeared to be carrying out a major role in the phagocytosis process for normal wild-type and homozygous calpain-1 KO cells. The live cell images and the Ca^{2+} signalling images suggested that there is a connection between the cell response and their Ca^{2+} levels during the phagocytosis process. This data implies that the neutrophil membrane projection and the completion of phagocytosis are related to the changes in the cytosolic free Ca^{2+} level.

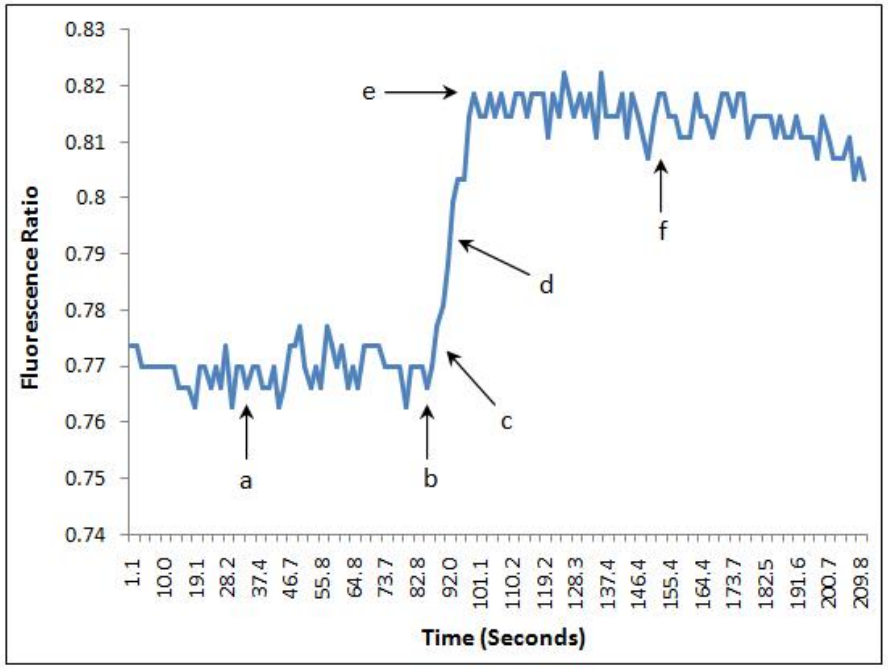
In normal wild-type neutrophils, the cytosolic Ca^{2+} levels rose during phagocytosis of the opsonised zymosan particles as represented by the Ca^{2+} signal images in Figure 8.4.2.1, 8.4.2.2 and 8.4.2.3. The constant levels of Ca^{2+} signals were recorded after the cells made contact with zymosan particles, and this was then followed by larger Ca^{2+} signals (shown by changes in the fluorescent intensity within the cell from blue to green) indicating the influx of Ca^{2+} in the cell cytosol (Figure 8.4.2.1, 8.4.2.2 and 8.4.2.3). From the live cell recordings, formation of the phagocytic ‘cup’ at the point of contact between the cells and the zymosan particles (Figure 8.4.2.1, 8.4.2.2 and 8.4.2.3) can be clearly distinguished just before the cells

complete their phagocytosis process. The series of responses showed by the cells suggested that the phagocytosis process was triggered by the rise in their Ca^{2+} level before a sequence of actions took place which lead to the engulfment of the zymosan particles. In addition, the response shown in Figure 8.4.2.3 illustrated a slightly different pattern of Ca^{2+} signals, which actually happens when the cell was in contact with more than one zymosan particle. At the end of recording, the cell proved to be capable of engulfing more than one particle before eventually closing their phagosome.

In general, the normal wild-type neutrophils were capable of phagocytosing when presented with the opsonised zymosan particles, and prior to that formed the phagocytic 'cup' at their points of contacts. An influx of Ca^{2+} in the cytosol appeared to be an important attribute for the cells to complete phagocytosis.

Normal Wild-type Neutrophils

A) Ca^{2+} signals in the normal wild-type neutrophil



B) Ca^{2+} signal images and live cell images

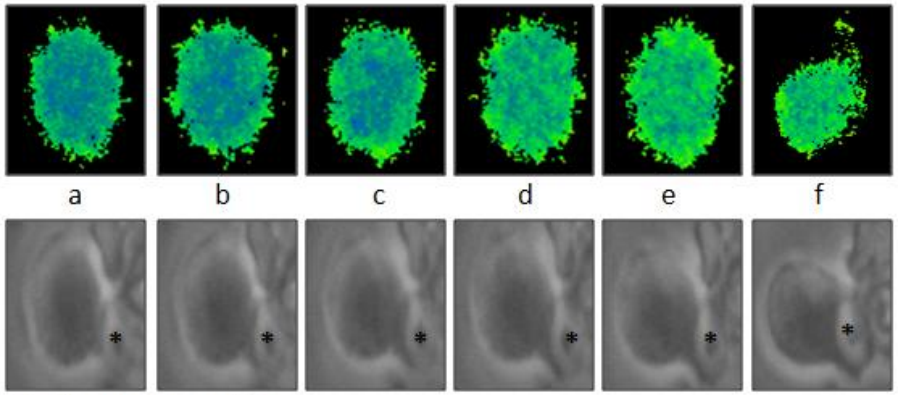
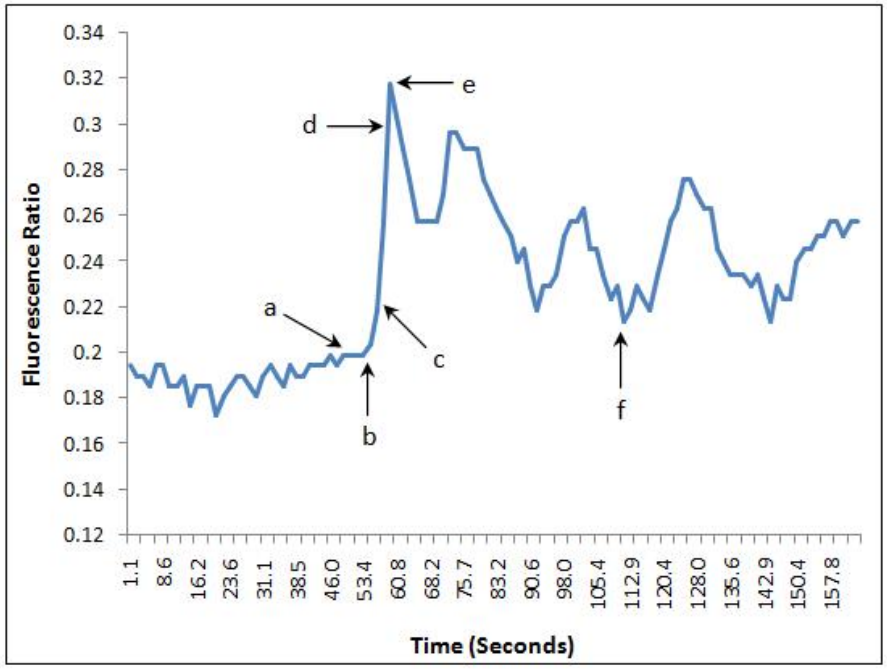


Figure 8.4.2.1: The graph showing Ca^{2+} signal in normal wild-type neutrophil after contact with opsonised zymosan. The graph in (A) shows the changes in Ca^{2+} signalling towards the zymosan particles and the reactions of a normal wild-type neutrophil recorded at different time points (in seconds). Images in (B) of the Ca^{2+} signal and live cell reactions which are indicated by the arrows in graph (A) portrays: (a) The formation of phagocytic 'cup' at 32 seconds. (b) The start of Ca^{2+} influx at 85 seconds. (c) The constant rise of Ca^{2+} signal at 90 seconds. (d) The influx of Ca^{2+} in the cell cytosol (changes in fluorescence intensity from blue to green) at 94 seconds. (e) The peak level of Ca^{2+} influx at 101 seconds. (f) The completion of phagocytosis at 153 seconds. The graph and images signify the connection between the influx of Ca^{2+} and the cells capability to phagocytose. This is recorded after the Ca^{2+} influx has reached its peak which would then promotes the completion of phagocytosis.

Normal Wild-type Neutrophils

A) Ca^{2+} signals in the normal wild-type neutrophil



B) Ca^{2+} signal images and live cell images

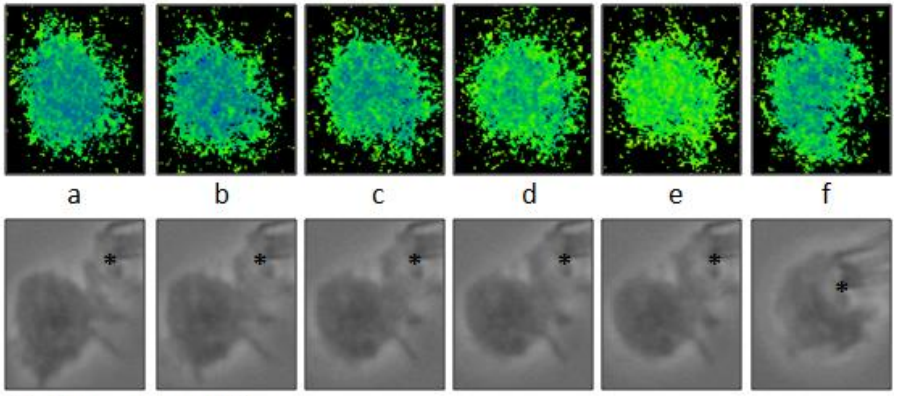
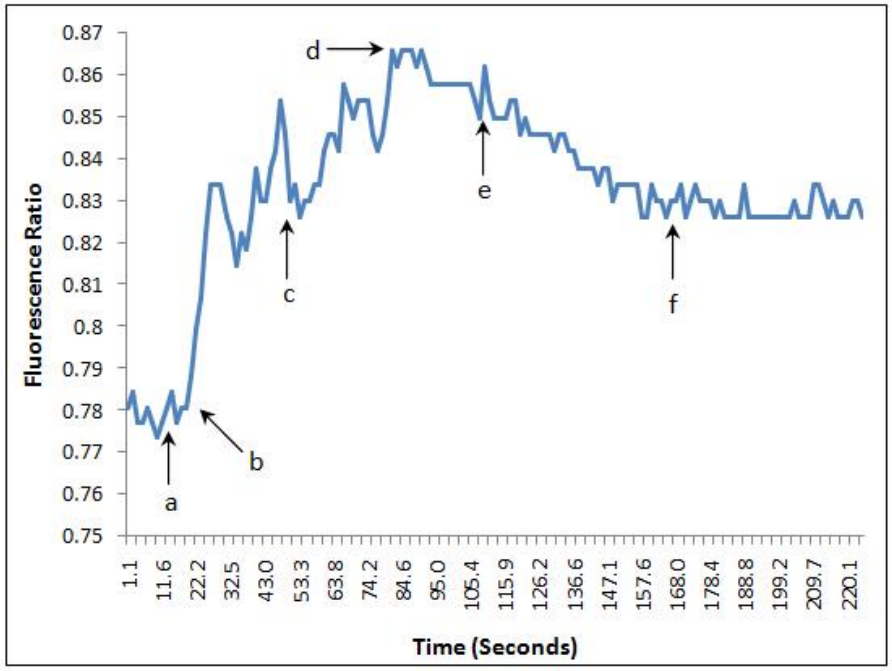


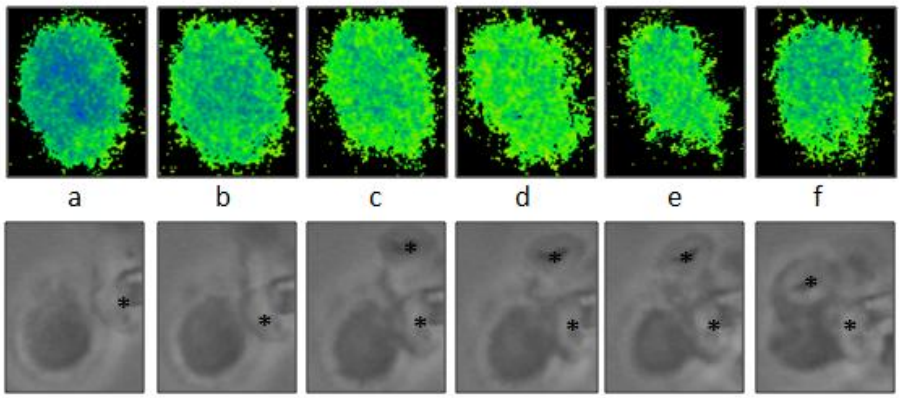
Figure 8.4.2.2: The graph of Ca^{2+} signal and the changes in Ca^{2+} level in normal wild-type neutrophil after making contact with the zymosan particles. The graph in (A) is another Ca^{2+} signal (in seconds) of a normal wild-type neutrophil and its reactions after making contact with the zymosan particles. Images in (B) of the Ca^{2+} signals and live cell reactions which are ndicated by the arrows in graph (A) represents: (a) Formation of the phagocytic 'cup' after 48 seconds. (b) The beginning of the Ca^{2+} level increase at 53 seconds. (c) The constant increase of Ca^{2+} level at 56 seconds. (d) The Ca^{2+} influx in the cytosol (changes in fluorescence intensity from blue to green) at 58 seconds. (e) The peak of the Ca^{2+} influx level at 59 seconds. (f) The phagocytosis process is completed at 109 seconds. The graph and images again showing the link between Ca^{2+} influx and the completion of phagocytosis which happened after the rise of cytosolic Ca^{2+} level in the cell reached its peak.

Normal Wild-type Neutrophils

A) Ca^{2+} signals in the normal wild-type neutrophils



B) Ca^{2+} signal images and live cell images



* = Zymosan particle

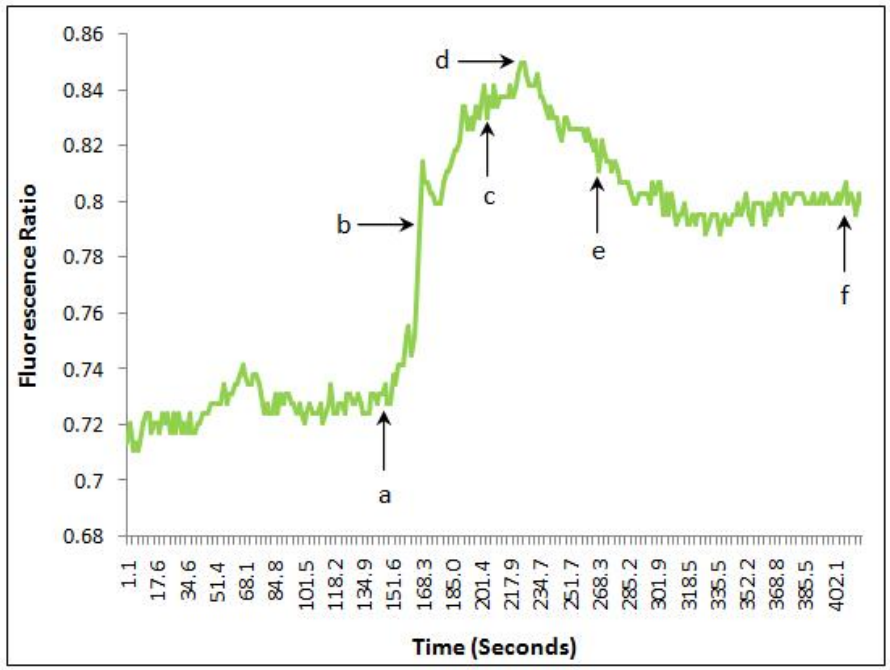
Figure 8.4.2.3: The graph of Ca^{2+} signal in normal wild-type neutrophils after making contact with opsonised zymosan. Graph (A) is the Ca^{2+} signal (in seconds) of a normal wild-type neutrophil and its responses after making contact with more than one opsonised zymosan. The Ca^{2+} signal and live cell images in (B) which are pointed out by the arrows in graph (A) shows: (a) The phagocytic 'cup' formed at 15 seconds. (b) The start of the Ca^{2+} level rise at 25 seconds. (c) The rise of Ca^{2+} level at 47 seconds, which also indicated the cytosolic Ca^{2+} influx (changes in the fluorescence intensity from blue to green). (d) The highest level of Ca^{2+} influx at 81 seconds. (e) Phagocytosis of the first zymosan particle is completed at 108 seconds. (f) The second zymosan is completely phagocytosed at 163 seconds. This reaction reaffirms the Ca^{2+} influx with the phagocytosis process, as well as demonstrating the capability of normal wild-type neutrophils to phagocytose more than one zymosan particle.

As for neutrophils from the homozygous calpain-1 KO animals, they also showed an increased Ca^{2+} level during phagocytosis of zymosan particles (Figure 8.4.2.4, 8.4.2.5 and 8.4.2.6). The cells demonstrated a similar gradual rise in their Ca^{2+} level as seen with the normal wild-type cells before showing a larger Ca^{2+} signal in their cytosol (Figure 8.4.2.4 and 8.4.2.5). However, one cell (Figure 8.4.2.6) did not demonstrate any signs of a gradual rise in its Ca^{2+} level after being presented with the zymosan particles, but only exhibited a large Ca^{2+} signal to indicate the influx of Ca^{2+} into the cytosol. The live cell recording showed the formation of phagocytic 'cup' at the point of contact between the cell and zymosan particle before eventually completing the phagocytosis process (Figure 8.4.2.4 and 8.4.2.5). Despite having a good Ca^{2+} signal, the cell in Figure 8.4.2.6 failed to form the phagocytic 'cup' and was not able to phagocytose. The video recordings of the cells in Figure 8.4.2.4 also proved that the homozygous calpain-1 KO neutrophil was capable of phagocytosis of more than one zymosan particle.

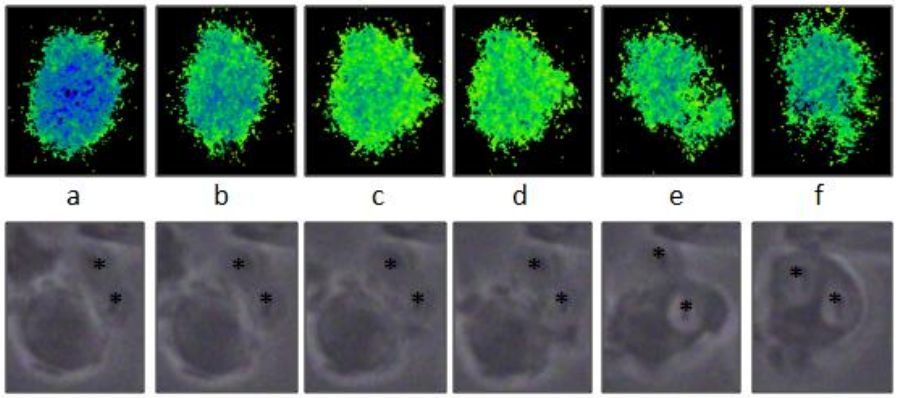
In general, the results have shown that the ability of the homozygous calpain-1 KO neutrophils to complete phagocytosis of the zymosan particles was still essentially intact. The detection of an influx of Ca^{2+} in the cytosol proved that their mechanisms for signalling Ca^{2+} are also functioning well, regardless of whether the cells completed phagocytosis or not. The same cells that have completed the phagocytosis process formed a phagocytic 'cup' at the point of contact with the zymosan particles. However, in contrast to the normal wild-type cells the 'cup' is only formed after the influx of Ca^{2+} has reached its highest level.

Homozygous Calpain-1 KO Neutrophils

A) Ca^{2+} signals in the homozygous calpain-1 KO neutrophil



B) Ca^{2+} signal images and live cell images

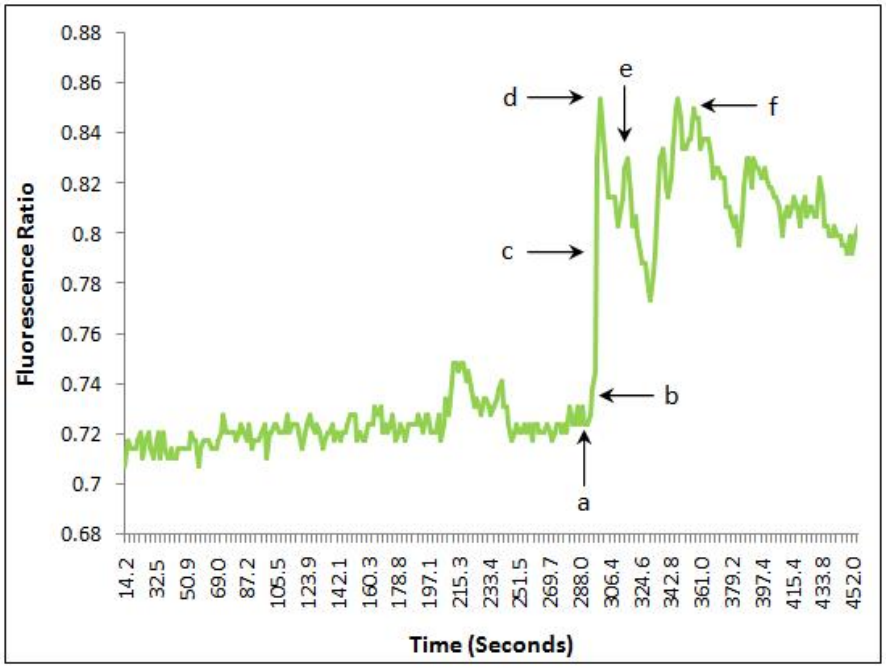


* = Zymosan particle

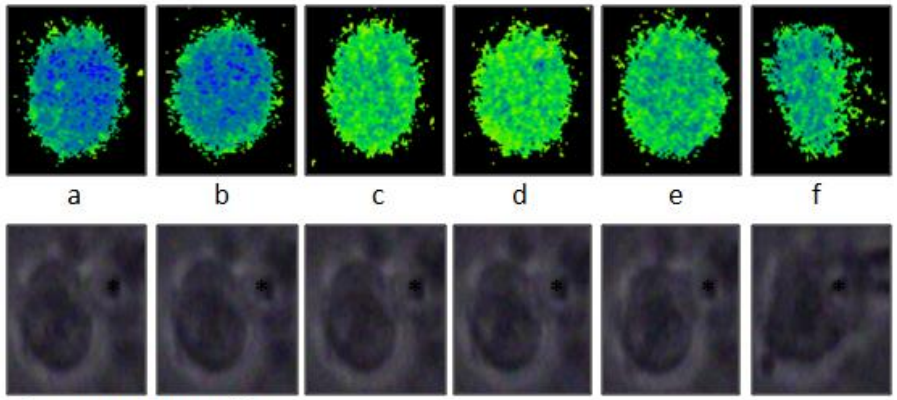
Figure 8.4.2.4: The graph of Ca^{2+} signal in homozygous calpain-1 KO cells after contact made with the opsonised zymosan particles. The graph in (A) is the Ca^{2+} signal (in seconds) recorded in a homozygous calpain-1 KO neutrophil and its reactions after making contact the zymosan particles. The Ca^{2+} signal and live cells images in (B) which are indicated by arrows in graph (A) represents: (a) The start of the Ca^{2+} level rise at 137 seconds. (b) The Ca^{2+} influx in the cytosol (changes in fluorescence intensity from blue to green) at 165 seconds. (c) The formation of phagocytic 'cup' at 209 seconds. (d) The peak of Ca^{2+} influx level at 224 seconds. (e) The phagocytosis process is completed at 264 seconds. (f) The cell appeared to phagocytose another zymosan particle at 413 seconds. This reaction provides evidence that the homozygous calpain-1 KO cell is capable of completing phagocytosis as well as signalling Ca^{2+}. In addition, this cell is also able to engulf more than one zymosan particles.

Homozygous Calpain-1 KO Neutrophils

A) Ca^{2+} signals in the homozygous calpain-1 KO neutrophil



B) Ca^{2+} signal images and live cell images

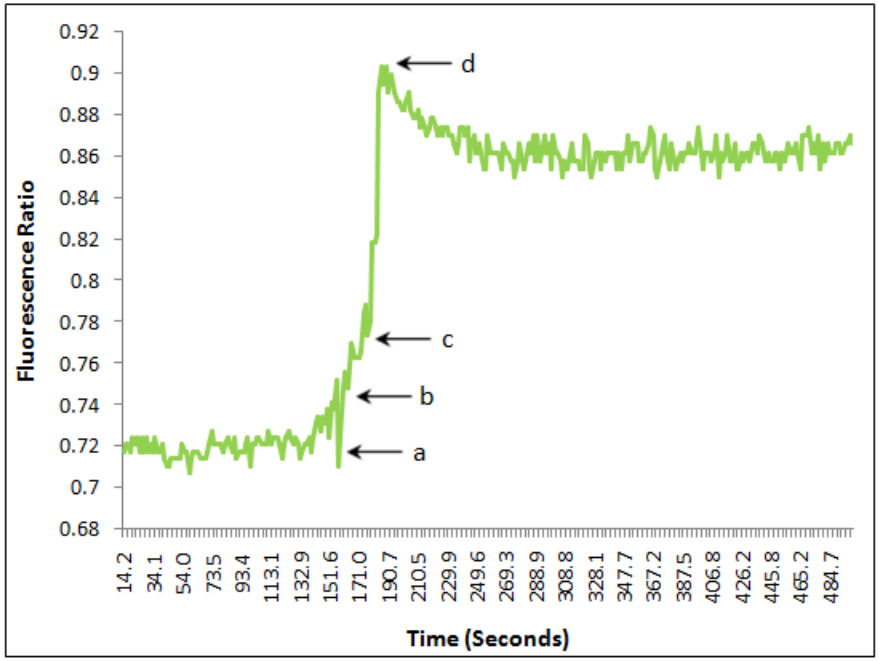


* = Zymosan particle

Figure 8.4.2.5: The graph of the Ca^{2+} signal from homozygous calpain-1 KO neutrophils after making contact with opsonised zymosan. Graph (A) is the Ca^{2+} signal (in seconds) of a homozygous calpain-1 KO neutrophil and its reactions after making contact with the zymosan particles. The Ca^{2+} signal and live cell reactions in (B) which are indicated by the arrows in graph (A) illustrates: (a) The start of Ca^{2+} influx at 292 seconds. (b) Constant rise of Ca^{2+} level at 294 seconds. (c) Cytosolic Ca^{2+} influx (changes in fluorescence intensity from blue to green) at 297 seconds. (d) The peak of Ca^{2+} influx at 300 seconds. (e) Phagocytic 'cup' formed at 311 seconds. (f) Phagocytosis is completed at 363 seconds. This reaction again showed the link between Ca^{2+} signal with phagocytosis in homozygous calpain-1 KO cell, and their ability to phagocytose. However, it also shows that the phagocytic 'cup' is formed after the Ca^{2+} level reaches its peak or during the rise of Ca^{2+} level as shown in Figure 8.4.2.4.

Homozygous Calpain-1 KO Neutrophils

A) Ca^{2+} signals in the homozygous calpain-1 KO neutrophil



B) Ca^{2+} signal images and live cell images

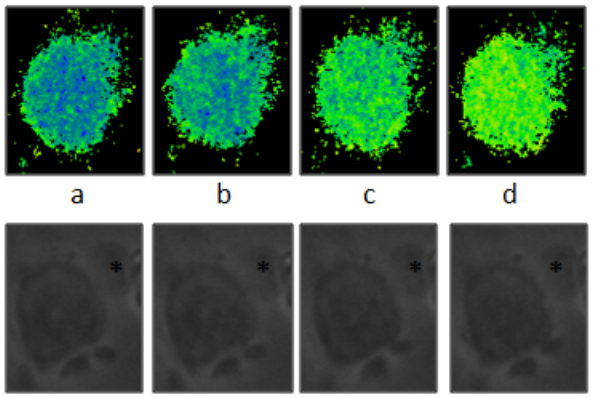


Figure 8.4.2.6: The graph of Ca^{2+} signal from a homozygous calpain-1 KO neutrophils after contact with the opsonised zymosan. The graph in (A) is another representation of Ca^{2+} signal (in seconds) taken from a homozygous calpain-1 KO neutrophil together with its response after making contact with the zymosan particle. The Ca^{2+} signal and live cell images in (B) indicated by the arrows in graph (A) shows: (a) The start of Ca^{2+} influx at 157 seconds. (b) The rising level of Ca^{2+} influx at 159 seconds. (c) The influx of Ca^{2+} in the cell cytosol (changes in fluorescence intensity from blue to green) at 178 seconds. (d) The peak Ca^{2+} level influx at 186 seconds. This result proved that the cell's mechanism for signalling Ca^{2+} is unharmed even in the absence of calpain-1. However, this cell has different reactions as compared to the other homozygous calpain-1 KO and normal wild-type cells. Despite having a good Ca^{2+} signal, this cell failed to phagocytose or to form the phagocytic 'cup'.

From images of the live cell recordings and changes in the Ca^{2+} levels, the reaction of the normal wild-type and homozygous calpain-1 KO cells towards the opsonised zymosan particles based on their reaction time points were analysed accordingly. In general, all of the cells that have phagocytosed the zymosan particle completed the whole process at different times starting from the moment the cells made contact with the zymosan. The average time taken by the normal wild-type cells to visibly formed their phagocytic 'cup' was recorded at 61.5 seconds (+/- 20 S.E.M), as opposed to the average time of 260 seconds (+/- 29.4 S.E.M) needed by the homozygous calpain-1 KO cells (Figure 8.4.2.7a). The time taken for the 'cup' formation after making contact with the zymosan particles showed a significant difference (p<0.05) between the homozygous calpain-1 KO and normal wild-type cells. However, there were no major differences with the time to complete phagocytosis once the phagocytic 'cup' had formed. On average, both cells had a comparable time from the 'cup' formation to phagosome closure, with 53.8 seconds (+/- 18.1 S.E.M) and 64.5 seconds (+/- 19.5 S.E.M) for normal wild-type and homozygous calpain-1 KO neutrophils respectively (Figure 8.4.2.7b).

The start of Ca^{2+} influx in the cytosol as shown by the cells also showed a significant difference (p<0.05) between them. The average time for Ca^{2+} influx in the normal wild-type cells was recorded at 69.3 seconds (+/- 15.5 S.E.M) as compared to the mean time of 195.3 seconds (+/- 30.8 S.E.M) by homozygous calpain-1 KO cells (Figure 8.4.2.8c). The early Ca^{2+} influx in normal wild-type neutrophils also meant that the cells could complete phagocytosis of the zymosan particles more rapidly than the homozygous calpain-1 KO neutrophils. This result suggested that although the Ca^{2+} signalling mechanisms in the homozygous calpain-1 KO neutrophils were unchanged, there was a defect with the cell's ability to perform the phagocytosis efficiently.

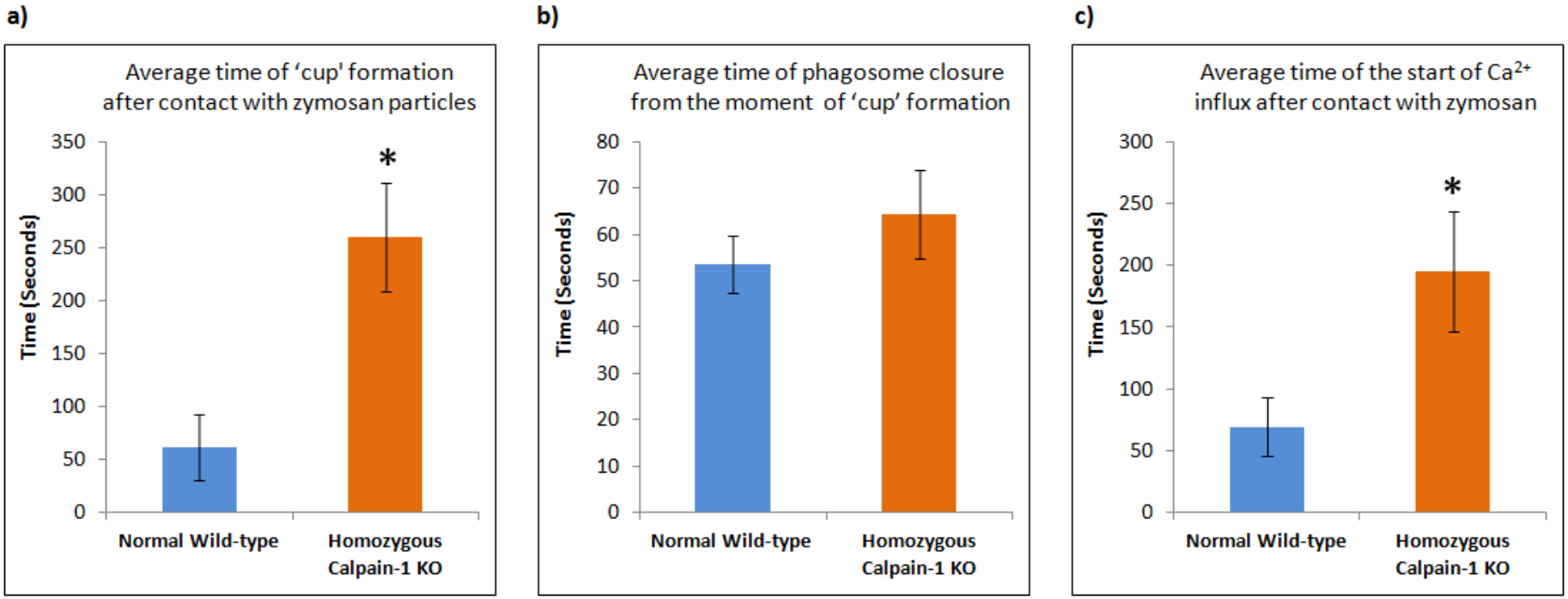
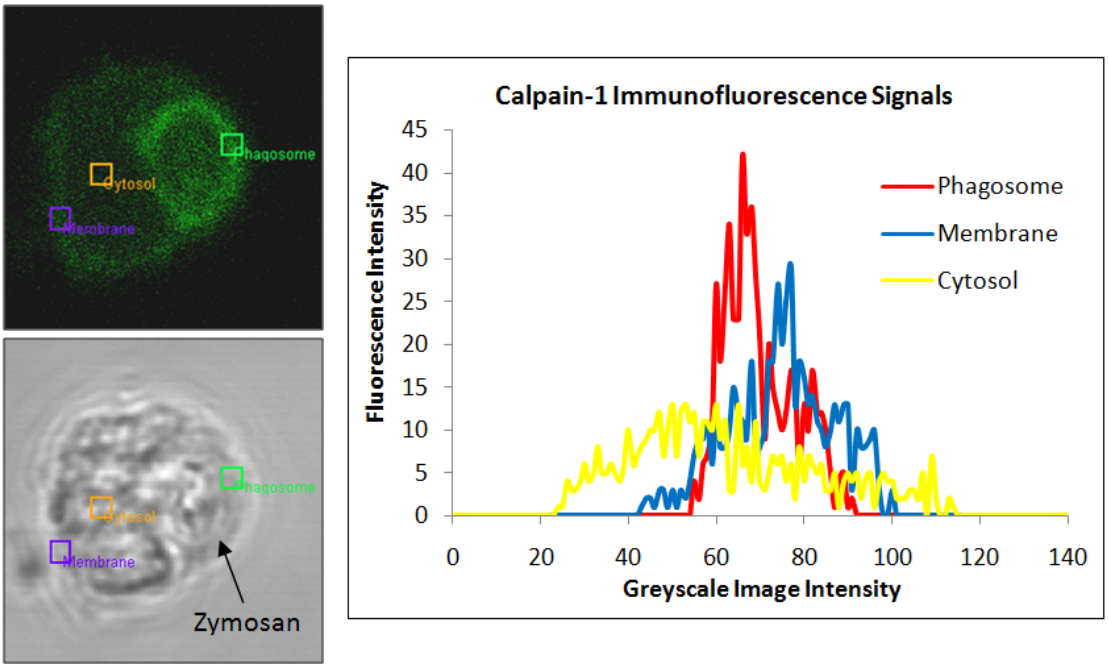


Figure 8.4.2.7: The graph of average time of the visible phagocytic 'cup' formation based on the live cell recordings of neutrophil. (a) The graph represents the average time of the visible phagocytic 'cup' formation based on the live cell recordings of normal wild-type and homozygous calpain-1 KO cells. The response time were significantly difference between the cell samples. (b) This graph shows that there is no significant difference in the average time taken by neutrophils of both normal wild-type and homozygous calpain-1 KO to completely engulf the zymosan particles or the first zymosan particles. (c) This graph shows the significant difference in the average time from the start of Ca^{2+} influx in normal wild-type as compared to homozygous calpain-1 KO neutrophils based on their Ca^{2+} signalling images after making contact with the opsonised zymosan particles. The data are reported as mean of ten different neutrophils and were compared by one-way analysis of variance. The comparisons were made using Student's *t*-test for unpaired values and set at $p<0.05$.

8.4.3. Calpain-1 Distribution in Neutrophils During Phagocytosis

In order to further investigate whether calpain-1 played a role in the phagocytosis process, the sub-cellular location of calpain-1 was established by using immunofluorescence staining. Calpain-1 was clearly detectable in normal wild-type cells lining the membranes of phagosomes which surround the zymosan particle (Figure 8.4.3.1a). The calpain-1 was also detected at the plasma membrane not in contact with zymosan particles. The level of the calpain-1 staining at this plasma membrane region was lower than at the phagosome but higher than in the cytosol (Figure 8.4.3.1a). This was consistent with a role for the calpain-1 during phagocytosis by mouse neutrophils. In calpain-1 deficient neutrophils, the antibody showed a faint staining on the plasma membranes and phagosome (Figure 8.4.3.1b). Once again, this result verified the effects of calpain-1 gene deletion and gives some indication about calpain-1 role in the neutrophil's phagocytosis process.

a) Normal Wild-type Neutrophils



b) Homozygous Calpain-1 KO Neutrophils

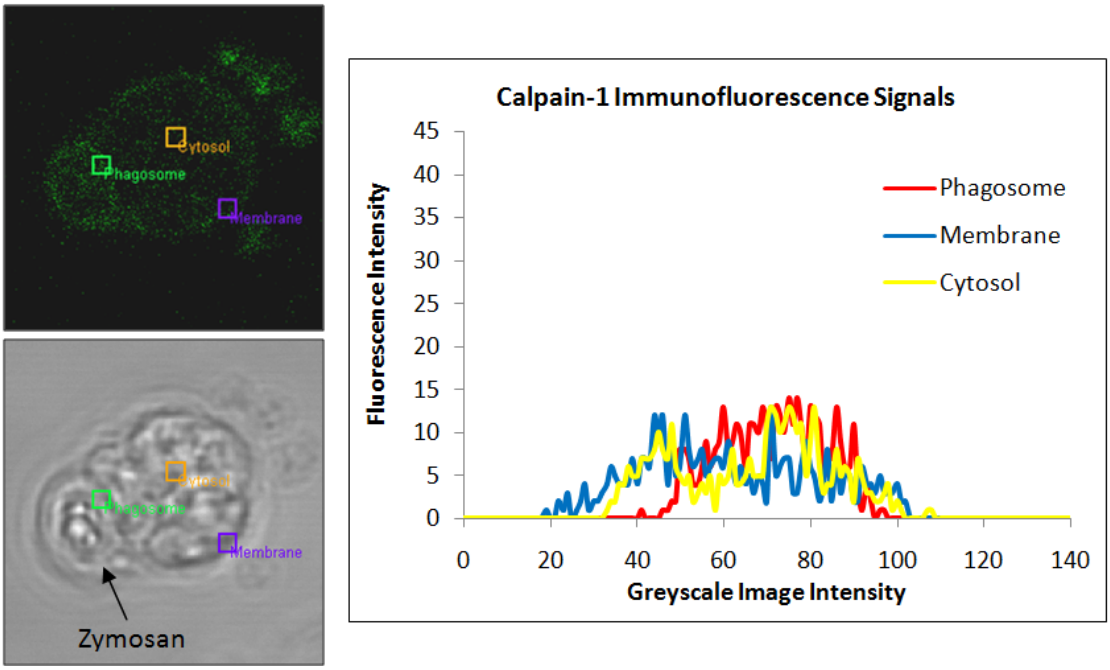


Figure 8.4.3.1: The pictures of calpain-1 immunofluorescence staining on normal wild-type and homozygous calpain-1 KO neutrophil. This figure represents the calpain-1 fluorescent signals on (a) normal wild-type and (b) homozygous calpain-1 KO neutrophil's taken using the confocal microscope. (a) The normal wild-type cells displayed higher calpain-1 signals on the phagosome around the zymosan particles and on the edge of their membrane, which is visibly marked in the fluorescence image. This is compared to the image in (b) of the homozygous calpain-1 KO neutrophils which showed weak fluorescence signals.

8.5. Discussion

The work in this chapter showed the capability of neutrophils from the normal wild-type and homozygous calpain-1 KO mice to complete phagocytosis, either when the cells were in suspension and permitted to phagocytose the opsonised zymosan particles through random contact, or when the cells were presented with the opsonised zymosan particles by micromanipulation. In either condition, neutrophils from both sets of samples were able of completing phagocytosis regardless of whether calpain-1 was present or not. The cell counts and analysis on the phagocytosis index has established that neutrophil populations of either the normal wild-type or homozygous calpain-1 KO mice completed the phagocytosis process with comparable percentages at every different time point. The images of neutrophils laden with the opsonised zymosan particles taken from the cell population sample proved that the ability of homozygous calpain-1 KO neutrophils to complete the phagocytosis process was not damaged.

However, by studying live cells during the phagocytosis process, variations in the cellular responses were observed with calpain-1 deficient neutrophils and found to have a subtle alteration. The Ca^{2+} signals showed by homozygous calpain-1 KO neutrophils after making contact with the zymosan particles proved to be altered as compared with the Ca^{2+} signalling patterns in the normal wild-type cells. Despite that, the onset of Ca^{2+} signalling between the normal wild-type and homozygous calpain-1 KO cells samples did show some variations. After making contact with the zymosan particle, the start of Ca^{2+} level increase in normal wild-type neutrophils occurred much earlier than the calpain-1 deficient neutrophils. This suggests that even though the mechanism for signalling Ca^{2+} within the cell cytosols

was still intact, the prolonged time needed by homozygous calpain-1 KO neutrophils means the absence of calpain-1 has affected their normal phagocytic function to some degree. This apparent delay in commencement of Ca^{2+} influx also had an impact on the time taken by the homozygous calpain-1 KO cells to complete the engulfment of a zymosan particle. The rapid influx of Ca^{2+} within the cytosol and their reactions towards the zymosan particles shown by normal wild-type cells suggests that their Ca^{2+} signals play a key role in controlling the phagocytosis process.

The reactions shown by the normal wild-type neutrophils also established that the phagocytic 'cup' was formed rather rapidly at the contact points with the zymosan particles and before the influx of Ca^{2+} reaches its peak. On average, the cells took shorter time to form their phagocytic 'cup' after making the first contact with the zymosan particles than the homozygous calpain-1 KO cells. As shown by the Ca^{2+} fluorescence images, homozygous calpain-1 KO neutrophils formed the 'cup' as the cytosolic Ca^{2+} level was rising or after the Ca^{2+} level has reached its maximum level. On the other hand, the average time taken by the cells, with or without calpain-1, to close their phagosome from the time of phagocytic 'cup' formation was comparable. However, the uptake of zymosan particles by normal wild-type cells showed significant difference as compared to the time needed by homozygous calpain-1 KO cells.

The rise in cytosolic free Ca^{2+} level accelerated the speed of the zymosan particle phagocytosis by human neutrophils, and by employing a calpain inhibitor, it was shown that the rapid uptake of zymosan particles have been inhibited (Dewitt and Hallett 2002). The result presented in this thesis suggested that the intracellular mechanisms and the signalling

pathways set off through the cross-linking reaction between the cells and zymosan particles may have been disrupted by calpain-1 gene deletion. Although the absence of calpain-1 did not stop the Ca^{2+} levels in the homozygous calpain-1 KO cells from rising, their delayed reactions after the Ca^{2+} influx implied that the cell's mechanism of action may have involved other forms of active enzymes that follow different pathways in order to complete the phagocytosis process as the normal wild-type cells would do.

The outcomes from these experiments suggests that the rise of cytsolic Ca^{2+} level in normal wild-type cells activates calpain-1 to cleave its potential substrates which act as tethers that hold the cell surface wrinkles together. The binding of the plasma membrane to the actin filament is released during phagocytosis and provides the extra membrane for cell expansion. Once the tethers are released, the cells will be able to extend their pseudopodia and rapidly engulf the zymosan particles. The presence of calpain-1 which is markedly located on the phagosomes around the zymosan particles in normal wild-type cells, and on the plasma membranes verified the involvement of this enzyme during phagocytosis. With the evidence that a large rise in cytosolic free Ca^{2+} levels accompanies phagocytosis (Marks and Maxfield, 1990), the location of the cytosolic Ca^{2+}-activated calpain-1 at the cell membrane, this suggests that calpain-1 is involved in the phagocytic event.

The immunofluorescence stains on homozygous calpain-1 KO neutrophils completing phagocytosis clearly showed the absence of calpain-1 within these cells. Thus suggests that after the influx of Ca^{2+} following the contact made with the zymosan particles, this cell may have to follow alternative pathways which involve different mechanisms or other enzymes in order to complete their phagocytosis process. This offers further evidence that without

calpain-1 involvement, the cell reaction is hindered and its rapid uptake of the zymosan particles has evidently been compromised. This deferred reaction shown by homozygous calpain-1 KO cells points to the longer time needed for intracellular interaction that would facilitate the cells ability to cleave the binding substrates between the membrane and actin filaments before extending their pseudopodia. The immunofluorescence staining implied that phagocytosis can still be achieved without the presence of calpain-1, and may perhaps be the result of an involvement of other calpain family members. Since western blotting showed that the antibody also recognised the small common subunit of calpain (calpain-4) and also gave lower cross reactivity with calpain-2 (see Chapter 5), it was possible that these subunits might have translocated to the phagosome and contributed to the ability of neutrophils to undergo phagocytosis. This may explain the ability of calpian-1 deficient mouse neutrophils to phaogocyose albeit at a slower rate. Therefore, this signifies calpain-1 as the enzyme which helps to accelerate the phagocytosis process whenever neutrophils made contact with foreign particles.

Chapter 9

General Discussion

9.1. Background

Neutrophils have been referred to as the first line of defence against infections, and are among the first defensive cell type to be recruited to the site of inflammation (Lew et al. 1985; Kruskal et al. 1986; Jaconi et al., 1991). This primary task is to remove and destroy invading bacteria by phagocytosis. The efficiency and rapidity of neutrophil action depends on their ability to change shape and transmigrate across the blood vessels walls, before undergoing chemotaxis to the target site (Theler et al. 1995). Neutrophils possess the C5a receptor for C5a that acts as chemoattractant to the site of inflammation, and CD11b/CD18 or β2-integrin for C3bi which participates in the acceleration of phagocytosis (Marks and Maxfield 1990; Dewitt and Hallett 2002; Dewitt et al. 2002). It has been recognized that changes in intracellular Ca^{2+} are fundamental to the behaviour and control of neutrophils during spreading and adhesion, chemotaxis migration and phagocytosis (Davies and Hallett, 1998; Hillson et al., 2006; Hillson and Hallett, 2007; Pettit and Hallett, 1997, 1998b).

One of the subject that has been difficult to determine is the correlation between neutrophil behaviour and their intracellular Ca^{2+} signalling processes. The cytosolic protease known as calpain is an enzyme that serves as one of the intermediary in the cells (Suzuki et al., 1992) and has been shown to cleave integrin molecules (Pfaff et al., 1999) and regulate the focal adhesion components through proteolysis of talin (Franco et al., 2004). Calpain-1 has been found to be the predominant isoform in resting neutrophils (Lokuta et al., 2003). It is hypothesized that the rise in cytosolic Ca^{2+} level activates resting calpain-1 and translocate to the plasma membrane, and as a result cleaves the actin binding protein that binds the actin filaments and integrin to permit shape changes (Tian et al., 2004).

In the work presented in this thesis, a homozygous calpain-1 KO mouse colony was successfully generated and enabled the roles of calpain-1 in neutrophil functions to be studied. This was achieved by establishing neutrophil's behaviour especially with respect to transmigration, adherence and spreading, chemotaxis, phagocytosis and the generation of Ca^{2+} signals. Therefore, the disruption caused by knocking-out calpain-1 to the behaviour of neutrophils can be clearly defined.

9.2. Results Presented in this Thesis

The behaviour of neutrophils from homozygous calpain-1 KO mice have contributed towards new understanding about the role of calpain-1 in regulating neutrophil functions. Using different methodologies and techniques to analyse neutrophil behaviour with regard to the absence of calpain-1, a number of important findings have been documented.

9.2.1. Successful Generation of the Homozygous Calpain-1 KO Mouse Colony

Success in generating the homozygous calpain-1 KO mouse colony was achieved through a breeding selection plan. The homozygous calpain-1 KO mice were viable and fertile, and survived in a "normal" environment. This proved that calpain-1 is not a critical factor for the reproductive progression in mice as compared to the embryonic fatality in the calpain-2 (Dutt et al., 2006) and calpain-4 deficient mice (Arthur et al., 2000; Zimmerman et al., 2000).

9.2.2. Spreading Impairment in Calpain-1 KO Neutrophils

Following fMLP stimuli, smaller spreading measurements means that homozygous calpain-1 KO neutrophils ability to spread has been disturbed. This happened despite the fact that the cells intracellular Ca^{2+} signalling was not damaged, and the cells comparable amounts of "membrane reservoir" for spreading. This pointed to the event that happened after the influx of Ca^{2+}, which relates to calpain-1 activation. Activation of calpain-1 in normal wild-type neutrophils could lead to the cleavage of linker proteins between the actin filament and the β2 integrin in order to permit cell spreading. The defect in calpain-1 deficient neutrophil spreading suggests that the cells might have utilized an alternative pathway to cleave the tether that restricts cell expansion. This could involve other types or form of enzymes such as calpain-4 or calpain-2 which are not as efficient as calpain-1.

9.2.3. Inter-species Variation in Neutrophil Reaction via IP_3 Uncaging

In contrast to human neutrophils which can spread upon the release of intracellular Ca^{2+} by IP_3 uncaging (Hillson et al., 2006; Pettit and Hallett, 1998a), mouse neutrophils were unable to spread even after a substantial Ca^{2+} signal. This effect may be a sign of the inter-species variation which is downstream to the IP_3 pathway. However, the ability of both cell types to spread after IP_3 uncaging and followed by multiple Ca^{2+} signals was rather interesting. This effect suggests the possibility of signalling mechanisms that correlate with the IP_3 pathway which would trigger Ca^{2+} influx before cell spreading. This opens up the possibility of studying the effect of IP_3 uncaging in mouse neutrophil and this is important if the work on calpain-1 KO neutrophils from the mouse to be translated into human study.

9.2.4. Slower Migration Pace in Calpain-1 KO Neutrophils during Chemotaxis

It has been reported earlier that neutrophils have decreased ability for directional migration after calpain-1 inhibition (Katsube et al., 2008; Lokuta et al., 2003). However, in this thesis it has been demonstrated that the ability of calpain-1 deficient neutrophils to undergo chemotaxis was still intact, but occurred more slowly. The capability of neutrophils to undergo chemotaxis after fMLP stimuli requires the engagement of β2 integrin with the extracellular matrix. It is shown in this thesis that homozygous calpain-1 KO neutrophils can undergo chemotaxis even if their leading membranes were facing the opposite direction to the chemoattractant gradient. Thus far, it is not clear whether calpain-1 has any influence in neutrophil "directional sense" during chemotaxis. However this effect signifies that calpain-1 facilitates neutrophil rapid chemotaxis via its cleavage action on the linker proteins between the actin filament and integrin. Once the neutrophil is liberated from the anchor, the cells are free to progress. Slower chemotaxis in calpain-1 deficient neutrophils indicates that the effect could be a result of intracellular inefficiency to cleave the linker proteins binding with β2 integrin. This effect is similar to the cleavage action in cell spreading, which suggests the involvement of other molecules or enzymes such as calpain-4 or calpain-2 to release the tether and a longer time required to liberate the cells before they could progress in chemotactic manner.

9.2.5. Phagocytosis Interruption in Calpain-1 KO Neutrophil

Engulfment of the C3bi opsonised zymosan by homozygous calpain-1 KO neutrophils proved that their phagocytosis ability was not damaged. Ca^{2+} signals generated in this cell

means that their signalling transduction downstream to the C3bi-Integrin engagement was also functioning. However, delays in the phagocytic 'cup' formation and onset of global Ca^{2+} influx suggested disruption during phagocytosis. Calpain has been shown to be active at the plasma membranes (Molinari and Carafoli, 1997), and is activated by Ca^{2+} at micromolar concentration between 3-50µM (Cong et al., 1989; Goll et al., 2003). The Ca^{2+} concentration persisted just underneath the membrane and within the folds of neutrophil's membrane wrinkles between 30 to 80µM (Brasen et al., 2010; Davies and Hallett, 1998). This signifies that the message carried by the C3bi opsonised zymosan particle through β2 integrin might have caused the rise in local Ca^{2+} level at their point of contact. The increase in local Ca^{2+} concentration was sufficiently high to activate calpain-1 and cleaves the linker proteins to release the cell membranes and forms the phagocytic 'cup'. This effect could then cause a larger influx of Ca^{2+} that activates more calpain-1. The mechanism that could trigger the Ca^{2+} influx is still being investigated, but the STIM1/Orai1 machinery has been implicated (Prakriya et al., 2006; Schaff et al., 2009; Steinckwich et al., 2011; Zhang et al., 2005).

The labelled β2 integrin in human neutrophils was abundant near the phagocytic 'cup' and available for facilitating phagocytosis (Dewitt and Hallett, 2002). This phenomenon could be an indication that activated calpain-1 is primarily attracted towards the β2 integrin location and largely cleaves the linker proteins between actin filament and β2 integrin in close proximity to that particular position in order to accelerate phagocytosis. In contrast to the formation of a phagocytic 'cup' in normal wild-type neutrophil before the influx of Ca^{2+}, the calpain-1 deficient neutrophils formed their phagocytic 'cup' as their Ca^{2+} concentration was reaching its peak. This suggests that calpain-1 deficient neutrophils might have followed a different but slower pathway which involves other unknown Ca^{2+} activated molecules to

complete phagocytosis. This suggests that delays in cleaving the linker proteins at the site of β2 integrin accumulation near the phagocytic 'cup' might have slowed down the course of action and decelerated the phagocytosis process in the cell.

9.2.6. Trans-endothelial Migration Defect in Calpain-1 KO Neutrophils

Transmigration defects in homozygous calpain-1 KO neutrophils *in vivo* and *in vitro* points toward the calpain-1 knocked-out effect. Neutrophil transmigration in the direction of the zymosan particles *in vivo*, or the fMLP *in vitro* indicates that the ability of calpain-1 deficient neutrophils have been disrupted. Although the interaction between β2 integrin and TNF-α has definitely enhanced the trans-endothelial migration process in normal wild-type neutrophils, this was not the case with calpain-1 deficient neutrophils. Ca^{2+} signals in calpain-1 deficient neutrophils undergoing phagocytosis verified that the cross-talk through β2 integrin was unharmed. Hence, the trans-endothelial migration results indicates a defect which is downstream to the increase in cytosolic free Ca^{2+} level and might have affected the β2 integrin engagement and possibly the upregulation of ICAM-1 after the TNF-α treatment. This could be explained by the disruption in the ability of calpain-1 deficient neutrophils to achieve their full spreading capacity, which suggests that the cells will have further difficulty to undergo the complex trans-endothelial migration process through the endothelial cells barrier. The trans-endothelial migration process is a complicated process which requires neutrophils not only to change their shape but also to cross the endothelial cells in either transcellular or paracellular routes (Ley et al., 2007). Thus, this implied that the defects due to lack of calpain-1 such as in cell spreading would be sufficient to compromise cellular interactions during trans-endothelial migration.

The trans-endothelial migration process engages with essentially the entire surface area of neutrophils in order to cross the endothelial cell barrier. This explains why the trans-endothelial migration capability in calpain-1 deficient neutrophils was more affected than the phagocytosis process which requires involvement of a much smaller cell surface area. The inefficiency of calpain-1 deficient neutrophils to cleave the linker proteins between the actin filament and β2 integrin would resulted in a prolonged response time for the cells to undergo trans-endothelial migration, and this maybe because of slower β2 integrin recycling (Lawson and Maxfield, 1995). The cells directed migration towards the fMLP gradient across the endothelial cell barrier gave further proof that their "directional sense" is functioning. Although the cell is liberated from their β2 integrin tether and could move freely, the disruption points to the speed at which calpain-1 deficient neutrophils release their tether for β2 integrin recycling during trans-endothelial migration. This indicates that the cells may need twice as much time compared to normal wild-type neutrophils to undergo the trans-endothelial migration process.

The complexity of trans-endothelial migration also means that there is numerous molecular interactions between β2 integrin on the surface of neutrophils and the surface molecules on endothelial cells. Neutrophils could undergo transmigration via paracelluar or transcellular route (Ley et al., 2007; Woodfin et al., 2010), but the mechanisms by which these events take place is still being discussed. However, β2 integrin has been identified as the integral part in both of the processes which could also be influenced by molecules such as the intercellular adhesion molecule-2 (ICAM-2), junctional adhesion molecule-A (JAM-A) and the platelet endothelial cell adhesion molecule-1 (PECAM-1) (Gane and Stockley, 2011; Woodfin et al., 2009). Whether neutrophils cross the endothelial cells barrier via complex

paracellular or transcellular process, flaws in releasing the β2 integrin tether would result in a trans-endothelial migration defect. Therefore, the outcomes presented in this thesis suggest that calpain-1 deficiency may possibly have caused the disruption in trans-endothelial migration and pointed to the role played by calpain-1 in regulating neutrophil functions through their cytoskeleton reorganization.

9.3. Future Prospects

From the results presented here, a number of important questions have been raised and suggest some interesting areas for future research.

1. The homozygous calpain-1 KO neutrophil recruitment *in vivo* and their trans-endothelial migration *in vitro* appeared defective. However, in order to understand these effects, a more physiological approach of inserting homozygous calpain-1 KO neutrophil into the normal wild-type mice can be employed.

2. Although their functions were disrupted, homozygous calpain-1 KO neutrophils were able to perform their various tasks despite lacking calpain-1. Adding calpain or calpain-1 inhibitor to the homozygous calpain-1 KO neutrophil could provide useful information with regards to the redundant cell signalling mechanism.

3. Accumulation of β2 integrin at the phagocytic 'cup' should attract activated calpain-1 to the site to unbind the cell's tether. Studying calpain-1 translocation during phagocytosis would explain the link between calpain-1 and its cleavage action with β2 integrin.

4. The analogous structural resemblance with calpain-2 and the structural association with calpain-4 mean that these enzymes might have roles in regulating neutrophil functions. Examining the presence of calpain-2 and calpain-4, and its role in homozygous calpain-1 KO neutrophils would present some useful answers about their functions.

9.3.1. "Recalling" Calpain-1 KO Neutrophils in Normal Wild-type

Generation of the calpain-1 KO mouse has provided the opportunity to immortalize cells from the animals for future research using different methodologies and techniques. In a collaboration work with the Myeloid Cell Biology Group at Cardiff Institute of Infection and Immunity, a novel immortalized homozygous calpain-1 KO cell line of the animal's stem cells has been generated. Using an established method, unlimited quantities of neutrophils can be generated by immortalizing the progenitor with estrogen-regulated Hoxb8 (ER-Hoxb8) by using cytokines to target expansions of committed progenitors (Wang et al., 2006). Lineage cells from the bone marrow from the animal's femurs were harvested and enriched with the biotinylated antibodies against the "lineage" antigens (CD5, CD45R (B220), CD11b, Anti-Gr-1 (Ly-6G/C), 7-4, and Ter-119 antibody). Initially, phoenix cells from human embryonic kidney (HEK293T) cell lines were transformed to enable MMLV (Moloney murine leukaemia virus) DNA packaging in capsids and mixed with the FuGene 6 transfection reagent. This FuGene 6 formed a complex with DNA and transports it into the cells. The pre-stimulated lineage cells were suspended in progenitor outgrowth medium (POM), stem cell factor (SCF) and 1μM β-estradiol. After 10 days, only the lineage cells that were infected with the MMLV-ER-Hoxb8 vector survived the antibiotic selection and cultured in POM with SCF and oestrogen. By day 10, a robustly growing cell culture called myeloid progenitor Hoxb8 (MyPH8-B6) cells were

produced. Neutrophils were generated by differentiating the MyPH8-B6 cells. The cells were resuspended in POM containing only SCF and granulocyte-colony stimulating factor (G-CSF). By the fourth day, majority of the cells had differentiated into functional neutrophils.

Using this technique to generate calpain-1 deficient neutrophils and the prospect of transfecting the cells with calpain-GFP construct (Larsen et al., 2008; Nuzzi et al., 2007), the same neutrophil recruitment experiments through intraperitoneal injection can be done. By injecting immortalized calpain-1 deficient neutrophils tagged with calpain-GFP into normal wild-type mice; the cells may be "recalled" by performing gastric lavage after injecting zymosan into the peritoneal cavity of the mouse. This would give a realistic representation of the ability of calpain-1 deficient neutrophils to transmigrate in a normal physiological system. This method can be reversed by injecting calpain-GFP tagged normal neutrophils into the homozygous calpain-1 mice. The number of neutrophils recruited through this procedure could be quantified, and a defect in trans-endothelial migration *in vivo* should show a lower calpain-1 deficient neutrophil count in the peritoneal cavity of normal wild-type mice.

9.3.2. Inhibiting Homozygous Calpain-1 KO Neutrophils

The data presented in this thesis demonstrated several disruptions in the functional ability of calpain-1 deficient neutrophils, but these cells were still capable of completing their tasks. Calpain was named after many discussions and refers to a papain-like cysteine protease that requires Ca^{2+} for its activity (Sorimachi and Suzuki, 2001). Various members of the "calpain superfamily" have structures that rather diverged, most likely to adapt to their

physiological functions and specific to the cells in which they function. The calpain-1 deficient neutrophils might have stopped functioning due to the calpain-1 knock-out effect, but it may then trigger a redundant cell biology mechanism which has a slower response. Multicellular organism have been studied for their developmental processes and in the case of redundancy between gene duplicates (Delattre and Felix, 2009). It has been proposed that reuse of similar cell biological mechanisms have evolutionary implications depending on the differential requirement in various cells. Thus, it is hypothesized that using calpain-1 inhibitors lacking the isotype specificity on homozygous calpain-1 KO neutrophils would eliminate the redundant cell signalling mechanism and stopped neutrophil functions. The differences in cell reactions of calpain-1 deficient neutrophils as compared to uninhibited homozygous calpain-1 KO neutrophils would establish the role of calpain-1 in regulating neutrophilic functions and possible effects of the redundant cell biology mechanism.

9.3.3. The Role of Calpain-2 and Calpain-4 in Neutrophils

The structured of calpain consists of either calpain-1 or calpain-2 in a heterodimeric formation with the smaller sub-unit calpain-4. Knocking-out calpain-1 suggested that it has diminishing effects on calpain-4 expression in the homozygous calpain-1 KO cells (Chapter 3). There is little known about the role of calpain-4 in neutrophils, and this might be a hint at the importance and the involvement of calpain-4 in regulating neutrophil functions. The lethal effect on the embryos, and the elimination of calpain-1 and calpain-2 activities in homozygous calpain-4 KO mice (Arthur et al., 2000) suggests the importance of calpain-4 in physiological functions. Thus, the diminishing presence of calpain-4 might also contribute towards the disruption in neutrophilic functions observed in this thesis. Although calpain-1

has been reported as the predominant isoform in neutrophil, calpain-2 was also implicated in neutrophil migration (Nuzzi et al., 2007). It was suggested that calpain-2 is one of the signalling components on the leading edges of the neutrophils which promotes their cellular polarization during chemotaxis (Nuzzi et al., 2007). In T lymphocytes, calpain-2 was reported to be involved with the release of lymphocyte function-associated antigen-1 (LFA-1) at the trailing edges and preserved in an active state by Ca^{2+} (Svensson et al., 2010). It has also been shown that calpain-2 regulates the membrane protrusions in fibroblasts which allow the cells to migrate efficiently (Perrin et al., 2006). Therefore, understanding calpain-2 and calpain-4 roles may provide valuable information that could have a significant impact in regulating neutrophil functions.

9.3.4. Collagen Induce Arthritis (CIA) in Calpain-1 KO Mice

Rheumatoid arthritis (RA) is a chronic disease that is generally characterized by the inflammation of the joints. The inflammatory reactions in such a condition involve the recruitment of white blood cells, which includes neutrophils, from the circulation into their target location. The generation of a homozygous calpain-1 KO mouse colony presents the opportunity to study the efficiency of calpain-1 deficient neutrophil recruitment in arthritis condition. Collagen induced arthritis (CIA) is a well established experimental model that has been applied in mice. By using collagen type II emulsified in complete Freund's adjuvant, arthritis is initiated by intradermal injection of the mixture into the hind paw of a mouse (Durie et al., 1994; Inglis et al., 2007; Williams, 2004). The injection incites an immune response to generate antibodies to the collagen type II which cause joint destruction involving cellular infiltration. This condition shares many similarities with the human RA.

Therefore, by using the same technique in the homozygous calpain-1 KO mice, the arthritis incidence of CIA can be analysed. In theory, trans-endothelial migration defect in calpain-1 deficient neutrophils should see lower numbers of neutrophils accumulating at the inducted knee joints as compared to the normal wild-type mice. For the histological assessment of arthritis, arthritic mice were sacrificed between 14 to 21 days after the disease onset, which is the ideal point to see the difference in neutrophil accumulation. Disruption in neutrophil migration in the homozygous calpain-1 KO mice could also be reflected by a smaller swelling measurement of their hind paws than the control subject. This could indicate the defect in neutrophil recruitment that may be caused by poor trans-endothelial migration. This would also allow quantification of neutrophil accumulation and gives a more accurate explanation on the defect which relates to the calpain-1 deficiency. It is hypothesized that in the RA condition, neutrophil accumulation in the homozygous calpain-1 KO mice will be less due to the trans-endothelial migration defect. Therefore, investigating the incidence of induced arthritis condition in calpain-1 deficient mice could give a clear answer on the importance of calpain-1 in regulating neutrophil responses in RA.

9.4. Proposed Model

It is suggested in the present work that the Ca^{2+}-activated calpain-1 plays a part in regulating neutrophil functions. The increase in Ca^{2+} level is the focal point for calpain-1 activation that allows neutrophils to achieve their utmost spreading capacity in order to accomplish effective trans-endothelial migration. The work presented here has enabled the proposal of a model to explain the role of calpain-1 in neutrophils (Figure 9.4.1).

A) Calpain-1 Role in Neutrophil Spreading and Trans-endothelial Migration

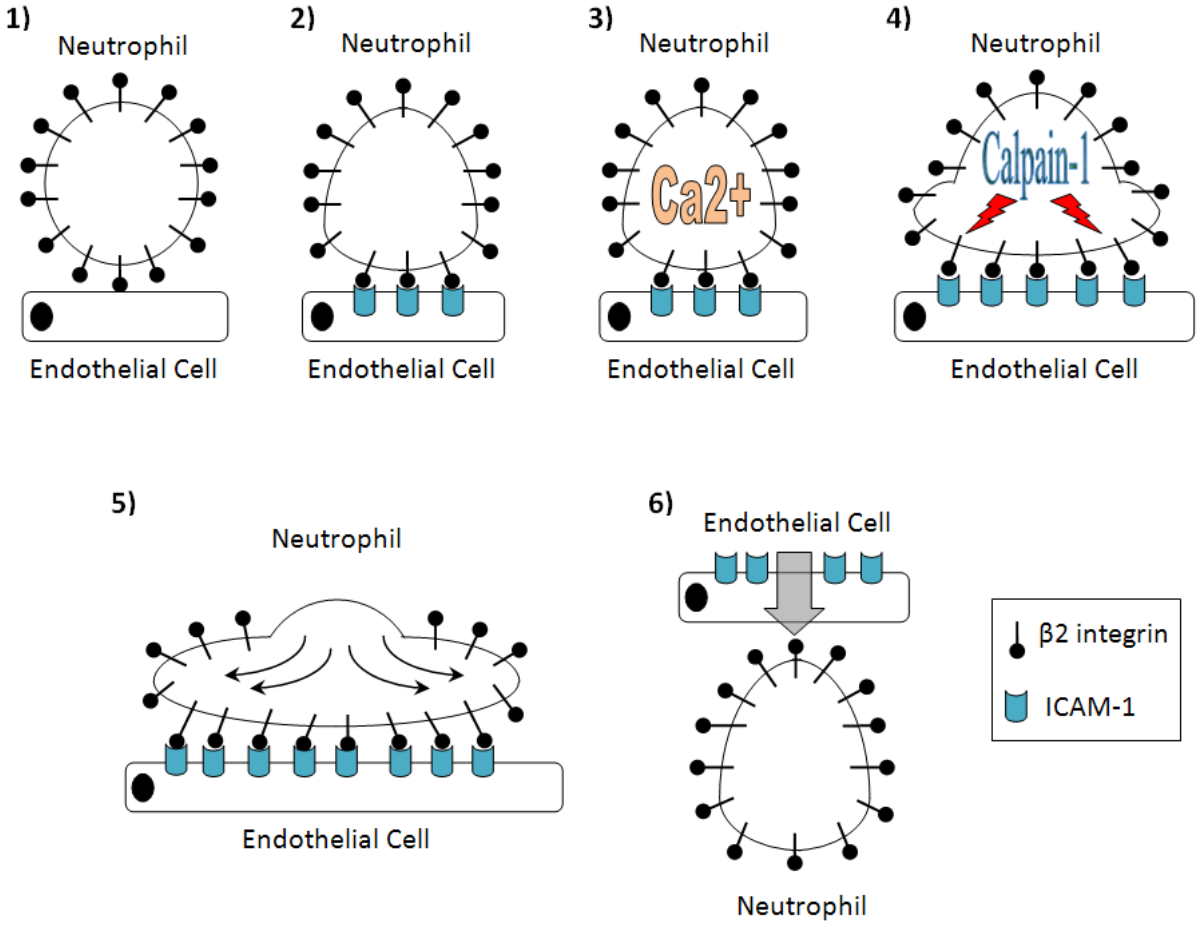


Figure 9.4.1: The proposed role of calpain-1 in neutrophil spreading and trans- endothelial migration. This diagram illustrates the proposed role of calpain-1 in neutrophil spreading and trans-endothelial migration. **(1)** The early step in recruiting circulating blood neutrophils involves slow rolling on the endothelial cells. **(2)** The release of an inflammatory agent in the form of TNF-α upregulates the expression of ICAM-1 which engages with the β2 integrin on the surface of neutrophil. This causes the cell to come to a halt and adhere onto the endothelial cells. **(3)** The presence of a chemoattractant such as C5a and the molecular interaction between β2 integrin and ICAM-1 increases the cytosolic free Ca^{2+} level in the neutrophil. **(4)** This subsequently activates the Ca^{2+} dependent calpain-1. As a result, the activated calpain-1 cleaves the linker proteins between β2 integrin and actin filaments that hold the cells in shape and restrict membrane expansion. This allows more β2 integrin to be engaged with ICAM-1. **(5)** By liberating the cell membrane from their "anchor" and gradually establishing more β2 integrin-ICAM-1 engagement, the neutrophil is able to expand and achieve maximum spreading capacity. **(6)** By means of excellent cell adhesion and spreading via β2 integrin and ICAM-1 engagement, the neutrophil is able to effectively complete trans-endothelial migration across the endothelial cell barrier.

9.5. Conclusion

The behaviour of neutrophils is complex and has a very significant impact on the inflammatory reactions. Therefore it is important to know how to control this behaviour in order to combat diseases related to inflammatory disorders. It follows the cascade of events which enable neutrophil to change their shape by liberating the cell membrane from the cytoskeleton anchorage. Even though this cellular reaction has not been fully understood, the genetic deletion of calpain-1 enzyme provides new ideas that help to understand the mechanism that orchestrates neutrophil behaviour. The presence of the calpain-1 enzyme on the membrane of spreading neutrophils, and during phagocytosis means that liberating the cells is associated with molecular interaction with β2 integrin by cleaving the tether. In conclusion, calpain-1 deficiency has resulted in defective neutrophil spreading which leads to poor trans-endothelial migration, and this could have a therapeutic potential in order to treat inflammatory disorders.

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Appendices

Appendix I - Cell Counting using a Haemocytometer

1. Ensure the cover-slip and haemocytometer are clean and grease-free (use alcohol to clean).
2. Moisten (with water or exhaled breath) and affix cover-slip to the haemocytometer.
3. Look for "Newton's Rings" which indicate that the cover slip has adhered via suction to the haemocytometer. Newton's Rings are seen as rainbow-like rings under the coverslip.
4. Mix equal volumes of 0.4% trypan blue stain and a well mixed cell suspension (e.g. mix 50µl trypan blue stain with 50 µl cell suspension).
5. Pipette trypan blue/cell mix (20µl) at the edge of the cover-slip.
6. Visualize the haemocytometer grid under the microscope, refer to Figure 1. Please note:
   i. Trypan Blue is a "vital stain"; it is excluded from live cells.
   ii. Live cells appear colourless and bright (refractile) under phase contrast.
   iii. Dead cells stain blue and are non-refractile.
   iv. To aid accuracy and consistency of cell counts use the system illustrated in Figure 2.
7. Count viable (live) and dead cells in one or more large corner squares and record counts.
8. Count more than one large corner square, and determine the mean number of cells/square
9. To calculate cell concentration per ml:
   a. Average number of cells in one large square x dilution factor* x 10^4* (see below)
      *dilution factor is usually 2 (1:1 dilution with trypan blue), but may need to further dilute (or concentrate) cell suspensions.
      *10^4 = conversion factor to convert 10-4ml to 1ml (refer to Figure 3 to view a diagram of the arrangement and dimensions)
   b. Divide this number by 2, since the depth of the haemocytometer is 0.2mm compared to most haemocytometers which are 0.1mm

Calculation of Cell Viability:

$$\frac{\text{No. of Viable Cells Counted}}{\text{Total Cells Counted}} \times 100 = \%\ \text{viable cells}$$

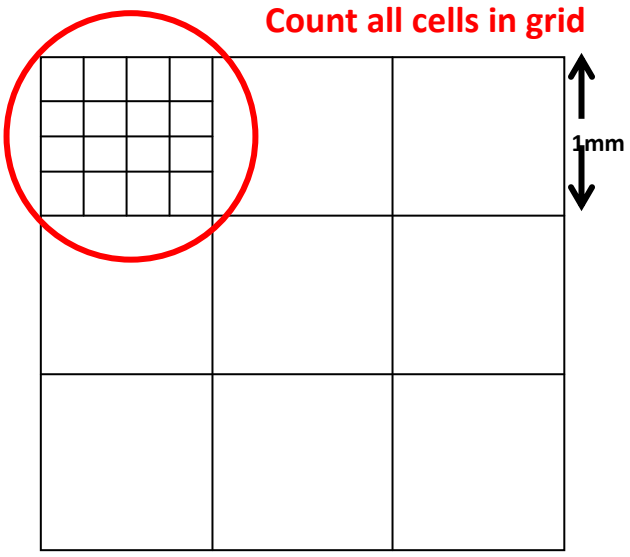


Figure 1: Appearance of the haemocytometer grid visualised under the microscope.

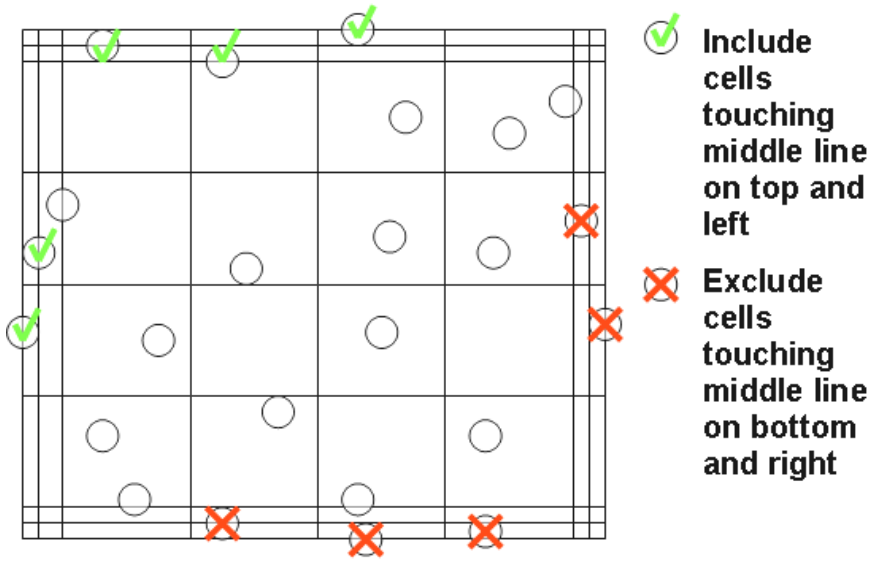
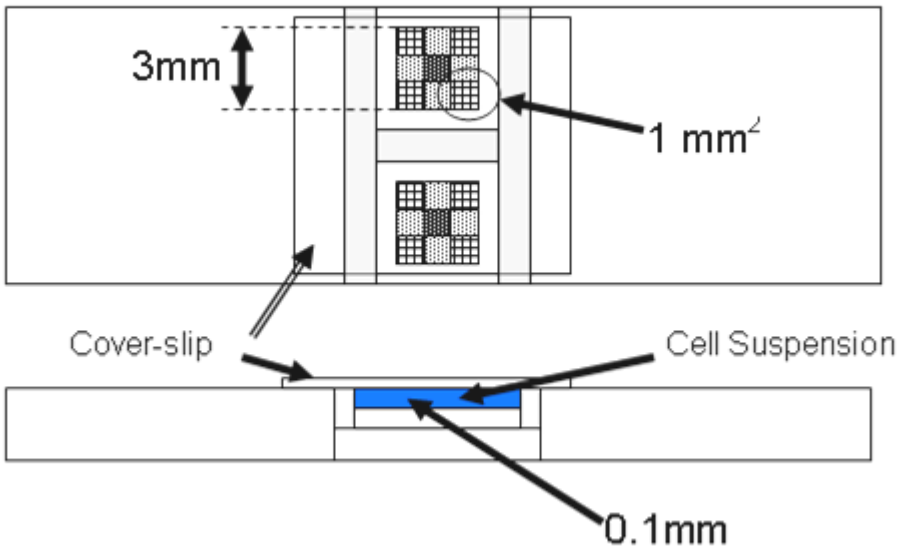


Figure 2: In order to ensure accuracy and consistency, count the cells within the large square and those crossing the edge on two out of the four sides.



Therefore, volume of 1 large corner square = 1 mm^2 x 0.1 mm = 0.1 cm x 0.1 cm x 0.01 cm = 10^{-4} cm^3 or 10^{-4} ml

Figure 3: Regular Haemocytometer arrangement and dimensions.

Appendix II – Protocol for Trans-endothelial Migration Assay

A. Transmigration Assay – Growing HUVECs

1. Good amount of HUVECs in DMEM/RPMI (about 20% coverage) were grown in the inserts in a 24 wells plate (non-stick plate)
   *Inserts were prepared in duplicate for untreated and treated HUVECs with TNF-α
2. DMEM/RPMI was added into the wells to compensate the medium in the inserts
3. The HUVECs (24 wells plate) was incubated for about 3-4 days
4. After 3-4 days incubation, medium for the designated inserts for TNF-α treated HUVECs was removed and TNF-α in fresh DMEM/RPMI was added
5. The plate was incubated for 30 minutes
6. Before the assay, the inserts with TNF-α was washed by twice aspirating the medium and adding fresh medium
   *Final concentration of the fresh medium in the inserts should be about 100 µl
7. The medium in non TNF-α treated inserts was aspirated and reduced to 100 µl
8. The medium in wells for TNF-α treated inserts was replaced with fresh DMEM/RPMI (to remove any TNF-α residues)
   *The inserts are ready for the assay (neutrophils are to be added into the inserts)

B. Transmigration Assay – Neutrophils Isolation, Counting & Staining

1. 0.5–1 ml of blood collected from mouse through cardiac puncture
2. Whole amount of blood is added to 50 ml of BSS
3. Samples were centrifuged for 2 minutes at 2000 rpm
4. Supernatant was carefully removed leaving only the suspended RBC pellet
5. The RBC pellet is lysed with 2-3 ml of distilled water for about 1-2 minutes
6. BSS was quickly added to make up a 50 ml solution
7. Samples were centrifuged again for 2 minutes at 2000 rpm
8. Supernatant was carefully removed leaving only the suspended pellet+neutrophils
9. 500 µl of tissue culture medium (DMEM/RPMI) was added to the pellet+neutrophils and counted using haemocytometer & cell counter (10 µl cells in 10 µl trypan blue/buffer)
10. About 100 µl of neutrophils was used in cytospin (3-5 minutes) for quantitative representation of the initial numbers of neutrophils and stained with Hemacolor Rapid Staining Kit, and pictures were taken.
    *The remaining pellets+neutrophils are to be used for the assay

C. Transmigration Assay – fMLP Solution

1. fMLP from stock of 1 mM in DMSO was used
   - For 1 µM final concentration - 1 µl of stock fMLP diluted in 1 ml of buffer/medium.
   - 0.5 µM fMLP was used for this assay
2. 5 µl of stock fMLP was diluted in 10 ml DMEM/RPMI
   *This 0.5 µM of fMLP solution is to be used as chemoattractant for the assay

D. Transmigration Assay – Adding Neutrophils Into the HUVECs, Counting & Staining

1. 100 µl of isolated neutrophils was added into each inserts and incubated for 30 minutes
2. The medium in all of the wells in the plates was aspirated (remove the entire medium)
3. 500 µl of the prepared fMLP solution was added into each wells
4. The plate was incubated for up to 4 hours and migration is monitored after every one hour without removing the inserts out
5. After 4 hours, the inserts were removed and migrated neutrophils in the wells were properly mixed (this can be done in non-sterile condition)
6. Migrated neutrophils were counted using haemocytometer & cell counter (10 µl cells in 10 µl trypan blue/buffer)
7. For quantitative representation, the remaining migrated neutrophils samples were fixed with 4% formaldehyde for about 30 minutes and put in cytospin (3-5 minutes)
8. Neutrophils were stained using Hemacolor Rapid Staining Kit and pictures were taken.

Appendix III – Protocol for Homozygous Calpain-1 KO Cells Immortalization

A. Preparation of pMXs-IP:FL-ER-Hoxb8 retrovirus for conditional-immortalisation of neutrophil precursors

1. Phoenix cells (a HEK293T, human embryonic kidney, cell line that has been transformed to enable it to package MMLV viral DNA in capsids) were incubated in 6 well plates (1.5-2x10^6 /well) in DMEM with 10% FCS, 1% penicillin & streptomycin and 1% glutamine (37°C, 5% CO_2) overnight.
2. The pMXs-IP:FL-ER-Hoxb8 viral DNA, was mixed with FuGene 6 transfection reagent (Roche) in DMEM.
3. The medium of the phoenix cells was aspirated.
4. 2ml DMEM with 10% FCS, 1% penicillin and streptomycin, and 1% glutamine was added.
5. A Fugene:DNA complex was formed by the addition of 6µl Fugene to 94µl of DMEM (the Fugene was added directly into the medium and not allowing any to touch the sides).
6. The Fugene:DNA is incubated for 5 minutes at room temperature.
7. Then 1µg of DNA was added and the mixture allowed to incubate at room temperature for at least 15 minutes.
8. The Fugene:DNA complex was then added to the phoenix cells and left to incubate (37°C, 5% CO_2) for 48 hours.
9. The replicate supernatants were harvested and polybrene (5 µg/ml) was added.
10. The supernatant filtered with a 0.45 µm filter.
11. The supernatants were stored at -80°C in appropriately sized aliquots.

B. Purification of lineage cells from mouse bone marrow and pre-stimulation for infection

1. Femurs from mice were cleaned and the marrow was flushed out with sterile MACS Buffer (0.5% (w/v) BSA, 5mM EDTA in PBS) using a syringe and a 25 gauge needle.
2. The cells were counted and line cells were enriched using the MACS murine lineage depletion kit (Miltenyi Biotec), following the company protocol.
    a. This involved depletion of bone marrow cells using the following biotinylated antibodies against the following:
        i. CD5
        ii. CD45R (B220)
        iii. CD11b
        iv. Gr-1, 7/4
        v. Ter-119
    - Followed by magnetic separation through a MACS column

3. The cells were counted and centrifuged (350 x g, 5 mins).
4. Resuspended in pre-stimulation medium (IMDM, 15% (v/v) FCS, 1% penicillin and streptomycin, 1% (v/v) glutamine, 10ng/ml IL-3, 20ng/ml IL-6, 25ng/ml SCF) at 10^6 /ml.
5. The cells were incubated (37°C, 5% CO_2) for 3 days.

C. Retroviral infection and cell propagation of pre-stimulated lineage cells

1. The wells of a 12 well plate were coated with 10µg/ml fibronectin (2 hours, 37°C).
2. The pre-stimulated line cells were counted and resuspened in progenitor outgrowth medium (POM) (OptiMem, 10% (v/v) FCS, 1% (v/v) penicillin and streptomycin, 1% (v/v) glutamine, 30 µM β-mercaptoethanol) with 10 ng/ml SCF and 1 uM β-estradiol, to a concentration of $2x10^6$/ml.
3. Cells ($5x10^5$ cells in 250 µl) were placed in each well and 850 µl of the viral vector, the empty vector or the mock transfected supernatant was added to each well.
4. The cells were centrifuged (1500 x g, 90 mins, 25°C).
5. POM with SCF and β-estradiol (3 ml) was added to each well and incubated (37°C, 5% CO_2) for 2 days.
6. After 2 days the replicates were pooled, centrifuged (350 x g, 5 mins) and resuspended in POM with SCF, β-estradiol and 1.5 µg/ml puromycin.
7. The cells were then allowed to grow over 10 days with regular replenishing of the medium.
8. When using C57BL/6 lin^- cells, only the cells infected with the pMXs-IP:FL-ER-Hoxb8 vector survived puromycin selection.
9. And on day 10 the puromycin was removed and the cells cultured in POM with SCF and oestrogen.
10. On addition of puromycin a large proportion of the cells die, but within 3 days growth is noticeable and by day 10 the cells produce a robustly growing cell culture.
11. These cells were named myeloid progenitor Hoxb8 from C57BL/6 (MyPH8-B6) cells.

D. Differentiation of MyPH8-B6 cells into Neutrophils

1. MyPH8-B6 cells were washed three times with sterile PBS (350 x g, 5 mins).
2. The cells were then counted and resuspened ($5x10^4 – 10^5$ cells/ml) in 10ml POM containing SCF+G-CSF (both at 20 ng/ml).
3. The cells were monitored and 5 ml medium was added to the cells everyday for four days.
4. By the fourth day the majority of the cells had differentiated into functionally mature neutrophils.

E. Retroviral infection of MyPH8-B6 cells

1. The undifferentiated cells were counted and $5x10^5$ cells were centrifuged (350 x g, 5 mins) and resuspended in 1.5 ml neat viral supernatant containing 10 ng/ml SCF and 1 uM β-estradiol.
2. The cells were added to a well of a sterile 6-well plate and centrifuged (1500 x g, 90 mins, 25°C).
3. After centrifugation, 2 ml of POM containing SCF and β-estradiol was added to each well and the plates incubated for 2 days (37°C, 5% CO_2).
4. After 2 days incubation, the cells were either selected with antibiotics or sorted with a MoFlo Legacy cell sorter (Beckman-Coulter), depending on the specific viral vector that was used to infect the cells.